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EDITORIAL

Immunoglobulin G4-related gastrointestinal diseases, are they immunoglobulin G4-related diseases?

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Abstract

In immunoglobulin G4 (IgG4)-related disease (RD), organ enlargement or nodular lesions consisting of abundant infiltration of lymphocytes and IgG4-positive plasma cells and fibrosis are seen in various organs. Although infiltration of many IgG4-positive plasma cells is detected in the gastric and colonic mucosa and major duodenal papilla of patients with autoimmune pancreatitis, it cannot be diagnosed as a gastrointestinal lesion involved in IgG4-RD, because none of the following is observed in these lesions: a mass-like formation; dense fibrosis; or obliterative phlebitis. Based on our review of the literature, there appear to be two types of IgG4-related gastrointestinal disease. One is a gastrointestinal lesion showing marked thickening of the wall of the esophagus and stomach, consisting of dense fibrosis with abundant infiltration of IgG4-positive plasma cells, which usually show submucosal spreading. The other is an IgG4-related pseudotumor occurring in gastrointestinal regions such as the stomach, colon, and major duodenal papilla, showing polypoid or mass-like lesions. Most solitary IgG4-related gastrointestinal lesions that are not associated with other IgG4-RD appear to be difficult to diagnose. It is of utmost importance to rule out malignancy. However, these lesions may respond to steroid therapy. To avoid unnecessary resection, IgG4-related gastrointestinal diseases should be considered in the differential diagnosis.

Key words: Immunoglobulin G4; Autoimmune pancreatitis; Gastritis; Colonic polyp; Ulcerative colitis

Core tip: Although the concept of immunoglobulin G4 (IgG4)-related gastrointestinal disease remains unclear, there appear to be two types of IgG4-related gastrointestinal disease. One is a gastrointestinal lesion showing marked thickening of the wall of the esophagus and stomach, consisting of dense fibrosis with abundant infiltration of IgG4-positive plasma cells, which usually show submucosal spreading. The other is an IgG4-related pseudotumor occurring in gastrointestinal regions such as the stomach, colon, and major duodenal papilla, showing polypoid or mass-like lesions. It is of utmost importance to rule out malignancy. To avoid unnecessary resection, IgG4-related gastrointestinal diseases should be considered in the differential diagnosis.

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INTRODUCTION

Immunoglobulin G4 (IgG4)-related disease (RD) is a recently recognized systemic condition characterized by elevated serum IgG4 levels and steroid responsiveness. IgG4-RD shows organ enlargement or nodular lesions consisting of abundant infiltration of lymphocytes and IgG4-positive plasma cells and fibrosis. IgG4-RD affects various organs such as the pancreas, bile duct, gallbladder, liver, salivary gland, lacrimal gland, retroperitoneum, and lymph nodes simultaneously or metachronously. IgG4-RD frequently presents both clinically and radiologically with findings that mimic a malignancy, resulting in unnecessary resection[1-4]. According to comprehensive clinical diagnostic criteria for IgG4-RD[4], IgG4-RD is diagnosed when there is a characteristic diffuse/localized swelling or mass in a single or multiple organs with elevated serum IgG4 levels, or there are histological findings of abundant infiltration of IgG4-positive plasma cells and lymphocytes, along with fibrosis.

Autoimmune pancreatitis (AIP) is a typical lesion of IgG4-RD, and the concept of IgG4-RD was proposed based on research on AIP[1,2]. Although it has been reported that infiltration of many IgG4-positive plasma cells was observed in the gastric mucosa, colonic mucosa, and major duodenal papillae of some AIP patients[5-10], it is questionable whether they are the lesions involved in IgG4-RD. To clarify IgG4-related gastrointestinal disease, this article reviews the published literature about the relationships between IgG4 and gastrointestinal diseases such as esophagitis, gastritis, colitis, and duodenal papillitis with abundant infiltration of IgG4-positive plasma cells. A PubMed database search, from 1990 to April, 2013, using the terms "autoimmune pancreatitis or IgG4-related" and "esophagus, duodenum, papilla, colon" identified 116 papers. Additional sources were identified by scanning the bibliographies of original and review articles.

IGG4-RELATED ESOPHAGEAL LESIONS

There have been two case reports of IgG4-related esophagitis[11,12]. In both cases, esophageal stricture with thickening of the esophageal wall evoked debilitating dysphagia and weight loss. Endoscopy showed esophageal stricture without a cancerous lesion. With a diagnosis of gastrointestinal stromal tumor based on endoscopic ultrasound-guided fine needle aspiration (one case) and because of concerns regarding a hidden malignancy (one case), esophageal resection was performed in both patients. On gross examination, the resected specimens showed an esophageal submucosal stricture with mucosal ulceration and wall thickening; histologically, they showed transmural chronic fibrotic inflammation with abundant infiltration of IgG4-positive plasma cells and lymphocytes and phlebitis. There was no evidence of other IgG4-RD. The post-operative serum IgG4 level was 138 mg/dL in one case. Both lesions are considered esophageal manifestations of IgG4-RD and should be called IgG4-related esophagitis. These lesions would probably respond to steroid therapy. Thus, IgG4-related esophagitis should be kept in mind in the differential diagnosis of unexplained esophagitis with stricture.

IGG4-RELATED GASTRIC LESIONS

It has been reported that infiltration of many IgG4-positive plasma cells was observed in the gastric mucosa in 33%-47% of AIP patients[10,13]. Shinji *et al*[14] and Uehara *et al*[15] also reported that IgG4-positive plasma cells were significantly more abundant in the gastric mucosa of AIP patients. Most of the infiltrated IgG4-positive plasma cells in the gastric mucosa disappeared in the biopsy specimen from the gastric mucosa after steroid therapy[16]. However, neither dense fibrosis nor obliterative phlebitis was observed in the gastric mucosa of AIP patients. Baez *et al*[17] reported a patient with AIP and IgG4-related sialadenitis who showed diffusely thickened (up to 1.4 cm) and nodular gastric mucosa with abundant infiltration of IgG4-positive plasma cells. The patient's serum IgG4 level was within the normal range (58 mg/dL), but the gastric lesion improved after steroid therapy. Kaji *et al*[18] reported an AIP patient (IgG4 level, 595 mg/dL) with multiple sporadic polyps in the gastric body with erosion and redness on the surface containing many infiltrated IgG4-positive plasma cells. On the other hand, two 3-cm-sized submucosal tumors that were laparoscopically wedge-resected showed histological findings of storiform fibrosis with abundant infiltration of lymphocytes and IgG4-positive cells (> 50/hpf), and they were reported as IGg4-related inflammatory pseudotumor of the stomach[19]. Both cases showed normal serum IgG4 levels and no evidence of other IgG4-RD[19]. Rollins *et al*[20] also reported a laparoscopically resected 5.6-cm IgG4-related fibrosclerosing pseudotumor of the stomach. Three cases with well-circumscribed, sclerosing nodular lesions of the stomach composed of fibrous tissue with abundant infiltration of IgG4-positive plasma cells were reported, and they were not associated with other IgG4-RD[21,22]. Fujita *et al*[23] reported a case with refractory gastric ulcers that worsened after successful *Helicobacter pylori* eradication therapy. The biopsy specimens taken from the ulcers showed abundant infiltration of IgG4-positive plasma cells (50/hpf). The patient's serum IgG4 level was elevated to 203 mg/dL, but he had no other IgG4-RD.

Bateman *et al*[24] reported a case of intractable gastric ulcer showing storiform fibrosis and abundant infiltration of IgG4-positive plasma cells (> 100/hpf). These reported lesions are considered IgG4-related gastric lesions. Anjiki *et al*[25] reported that gastric emptying assessed by

the carbon 13 acetate breath test was impaired in AIP patients and improved to the reference range after steroid therapy, and they suggested that the stomach might be a target organ of IgG4-RD.

IGG4-RELATED MAJOR DUODENAL PAPILLARY LESIONS

It has been reported that the duodenal major papilla is swollen in 41%-65% of AIP patients[26-28]. Abundant infiltration of IgG4-positive plasma cells is reportedly detected in 55%-80% of AIP patients[8,10,26,27]. Both a swollen major papilla and abundant infiltration of IgG4-positive plasma cells have shown improvement after steroid therapy[8,29]. In the resected pancreas of AIP patients, lymphoplasmacytic inflammation with many IgG4-positive plasma cells was detected in the major duodenal papilla connected to the head of the pancreas; thus, IgG4 immunostaining of biopsy specimens obtained from the major duodenal papilla might be useful to support the diagnosis of AIP[8,26,27,30,31]. Hisa *et al*[32] reported a resected case of a lymphoplasmacytic granuloma with abundant IgG4-positive plasma cells localized to the major duodenal papilla. The case was not associated with other IgG4-RD. This lesion is considered to be an IgG4-related pseudotumor localized to the major duodenal papilla.

IGG4-RELATED COLONIC LESIONS

Although infiltration of many IgG4-positive plasma cells is occasionally detected in the colonic mucosa of AIP patients, dense fibrosis or obliterative phlebitis was not observed in the lesion[1,5-7,21,33]. Although Ravi *et al*[34] suggested that inflammatory bowel disease might represent an extrapancreatic manifestation of AIP, in general, conventional AIP (type 1 AI) is rarely associated with ulcerative colitis (UC)[2,35]. IgG4-positive plasma cell infiltration is sometimes detected in the colonic mucosa of UC patients[36-40], but the mechanisms underlying IgG4-positive plasma cell infiltration in the colonic mucosa of UC patients are unknown. Matsui *et al*[41] reported a case of an AIP patient with a colonic polyp (ascending colon) containing many IgG4-positive plasma cells[42] who developed colonic polyposis (descending colon) containing many IgG4-positive plasma cells 1 year after complete remission of AIP with steroid therapy. The polyposis was markedly reduced with re-administration of steroids. They suggested that enhanced T helper type 2 responses to intestinal microflora may underlie the immunopathogenesis in patients with IgG4-RD[43]. Well-circumscribed sclerosing nodular lesions of the cecum and sigmoid colon composed of hyalinized fibrocollagenous tissue with abundant infiltration of IgG4-positive plasma cells were reported, and the two cases had no other IgG4-RD[21]. These polypoid or nodular lesions appear to be IgG4-related colonic lesions.

IGG4-RELATED INFLAMMATORY PSEUDOTUMOR OF AN ILEAL CONDUIT

An ill-defined, fibrotic, tumor-like mass, histologically showing fibrosis with infiltration of lymphocytes and IgG4-positive plasma cells and marked obliterative phlebitis, occurred in an ileal conduit created as part of surgery for urinary bladder cancer[44].

DISCUSSION

IgG4-RD shows organ enlargement or nodular lesions consisting of abundant infiltration of lymphocytes and IgG4-positive plasma cells and fibrosis in various organs simultaneously or metachronously[3,4]. The first International Symposium on IgG4-RD held in 2011 suggested that the term "IgG4-related disease" aptly recognizes the ubiquity of IgG4 within involved organs, and proposes a style that employs "IgG4-related" as a prefix to the organ system affected and pathological guidelines for the diagnosis of IgG4-RD[3,45]. The diagnosis of IgG4-RD rests on the combined presence of the characteristic histopathological appearances and increased number of IgG4-positive plasma cells. A histologically high suspicion of IgG4-RD requires the presence of at least two of three characteristic histological features including (1) dense lymphoplasmacytic infiltration; (2) fibrosis, usually storiform in character; and (3) obliterative phlebitis. The IgG4 counts required for the diagnosis differ among affected organs, ranging from 10 to 200 cells/hpf. The diagnosis of IgG4-RD requires considering both histopathological findings and clinical information such as elevated serum IgG4 levels, other organ involvement that is consistent with IgG4-RD, and effective response to steroid therapy[45].

Comprehensive clinical diagnostic criteria for IgG4-RD[4] were proposed in 2011. In the criteria, IgG4-RD is diagnosed when there is a characteristic diffuse/localized swelling or mass in a single or multiple organs with elevation of serum IgG4 levels or IgG4-related histological findings. However, the concept of IgG4-related gastrointestinal diseases was not included as objects of the criteria. It is unclear whether IgG4-related gastrointestinal diseases exist or what gastrointestinal lesions are regarded as IgG4-RD. To clarify these questions, this review of IgG4-related gastrointestinal diseases, the first of its kind, was conducted.

Infiltration of many IgG4-positive plasma cells is detected in the gastric and colonic mucosa and the major duodenal papillae of some AIP patients, but none of the following are observed in these lesions: a mass-like formation; dense fibrosis; or obliterative phlebitis[5-10]. They cannot be diagnosed as gastrointestinal lesions involved in IgG4-RD, because, as in many other organ systems, increased IgG4-positive plasma cells do not mean the disease is one of the family members of IgG4-RD. At this point, both the clinical finding of mass forming and histological finding of abundant infiltration of IgG4-

positive plasma cells with fibrosis would appear to be necessary to make the diagnosis of IgG4-related gastrointestinal diseases.

IgG4-related pseudotumors have been reported in several organs, such as the liver and lung[46-48]. On review of these papers, there appear to be two types of IgG4-related gastrointestinal disease. One is a gastrointestinal lesion showing marked thickening of the wall of the esophagus[11,12] and stomach[17,23,24], consisting of dense fibrosis with abundant infiltration of IgG4-positive plasma cells, which usually show submucosal spreading. The other is an IgG4-related pseudotumor occurring in gastrointestinal regions such as the stomach[18-22], colon[21,42], and major duodenal papilla[32], showing polypoid or mass-like lesions. We currently consider these lesions to be IgG4-related gastrointestinal diseases. However, this is the first review of a few cases of IgG4-related gastrointestinal diseases; further studies should be conducted to confirm this concept.

Most solitary IgG4-related gastrointestinal lesions that are not associated with other IgG4-RD appear to be difficult to diagnose. It is of utmost importance to rule out malignancy. However, these lesions may respond to steroid therapy. To avoid unnecessary resection, IgG4-related gastrointestinal diseases should be considered in the differential diagnosis.

CONCLUSION

The concept of IgG4-related gastrointestinal disease remains unclear due to its rarity. There appear to be some IgG4-related gastrointestinal lesions that present with a mass-like lesion consisting of abundant infiltration of IgG4-positive plasma cells and lymphocytes and fibrosis.

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REVIEW

Pathophysiology, epidemiology, classification and treatment options for polycystic liver diseases

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Abstract

Polycystic liver diseases (PLD) represent a group of genetic disorders in which cysts occur in the liver (autosomal dominant polycystic liver disease) or in combination with cysts in the kidneys (autosomal dominant polycystic kidney disease). Regardless of the genetic mutations, the natural history of these disorders is alike. The natural history of PLD is characterized by a continuous increase in the volume and the number of cysts. Both genders are affected; however, women have a higher prevalence. Most patients with PLD are asymptomatic and can be managed conservatively. Severe symptoms can affect 20% of patients who develop massive hepatomegaly with compression of the surrounding organs. Rrarely, patients with PLD suffer from acute complications caused by the torsion of hepatic cysts, intraluminal cystic hemorrhage and infections. The most common methods for the diagnosis of PLD are cross sectional imaging studies. Abdominal ultrasound and computerized tomography are the two most frequently used investigations. Magnetic resonance imaging is more sensitive and specific, and it is a valuable test for patients with intravenous contrast allergies or renal dysfunction. Different treatment modalities are available to physicians caring for these patients. Medical treatment has been ineffective. Percutaneous sclerotherapy, transarterial embolization, cyst fenestration, hepatic resection and liver transplantation are indicated to specific groups of patients and have to be tailored according to the extent of disease. This review outlines the current knowledge of the pathophysiology, clinical course, diagnosis and treatment strategies of PLD.

Key words: Polycystic liver disease; Hepatic; Epidemiolgy; Classification; Therapy; Genetic

Core tip: The management of patients with symptomatic polycystic liver disease is challenging. Among several treatments options, the most common interventions are: percutaneous cyst aspiration, fenestration, hepatic resection and liver transplantation. There is no consensus on the best treatment options and the optimal timing for interventions in symptomatic patients. In vision of these limitations, we reviewed the most recent literature and present a comprehensive article on this topic.

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INTRODUCTION

The association between polycystic liver disease (PLD) and autosomal dominant polycystic kidney disease (ADPKD) was described for the fist time by Bristowe in 1856[1,2]. Initially, it was thought that PLD could develop only in the context of ADPKD[3]. The notion that isolated PLD might be a separate condition was proposed in the 1950's[4]. In 2003, a linkage analysis of eight Finnish families confirmed that PLD is genetically distinct from ADPKD[5]. Asymptomatic patients usually do not require any intervention[6]. In some patients, massive hepatomegaly can cause pain or compression of the adjacent gastrointestinal organs, vasculature, and diaphragm. This can have a significant effect on patients' quality of life and performance status[6,7]. For these patients, the main aim is to reduce their symptoms by decreasing the liver volume[8-10]. Current surgical options include open or laparoscopic cyst fenestration with or without hepatic resection and orthotopic liver transplantation (OLT). Significant advances in surgical techniques have improved the outcomes of PLD patients. However, the selection of the appropriate approach remains a clinical challenge, and there is no consensus on the optimal timing and what represents the best therapeutic modality.

INCIDENCE AND GENETICS

ADPKD affects up to 0.2% of the general population[11]. On the other hand, isolated PLD has prevalence of less than 0.01%[12]. Both ADPKD and PLD are autosomal dominant and 75%-90% of patients with ADPKD have associated PLD[13]. In humans, PLD has been linked to mutations of four genes. Two genes (*PKD1*, locus 16p13.3, encoding polycystin-1 and *PKD2*, locus 4q21, and encoding polycystin-2) are predominantly associated with renal disease and less frequently with PLD. *PKD1* mutations are more common and account for 85%-90% of the cases, whereas mutations in *PKD2* affect approximately 10%-15% of patients[11]. The remaining two mutations (PRKCSH, locus 19p13.2, encoding the protein kinase C substrate 80K-H or hepatocystin and SEC63, locus 6q21, encoding the Sec63 protein) are linked only to the development of PLD[11]. However, these mutations explain just 25% to 40% of cases of PLD[14,15]. Comparative characteristics between ADPKD and PLD are summarized in Table 1.

PATHOPHYSIOLOGY

Malformation of the hepatic ductal plate and cilia of cholangiocytes is the main characteristic linked to the pathophysiology of PLD (Figure 1).

DUCTAL-PLATE MALFORMATION

The ductal plate is the anatomical template for the development of the intra-hepatic bile ducts[16]. Normal bile ducts arise from the ductal plate through a complex sequence of growth and apoptosis. Complexes of disconnected intralobular bile ductules (von Meyenburg complexes) are retained because they do not undergo apoptosis in PLD[10]. As a consequence, multiple cysts arise from progressive dilatation of these abnormal ductules[17-19] that display the same epithelium and structures of functioning cholangiocytes[20,21].

Table 1 Comparative epidemiological and genetic mutation characteristics of autosomal dominant polycystic kidney disease associated polycystic liver disease and isolated polycystic liver disease

Characteristics	ADPKD associated PLD	Isolated PLD
Prevalence	0.20%	< 0.01%
Type of inheritance	AD	AD
Gene mutated	*PKD1; PKD2*	*PRKCSH; SEC63*
Encoded product	Polycystin-1; Polycystin-2	Hepatocystin; Sec63 protein
Chromosome locus	216p13.3; 4q21	19p13.2; 6q21

AD: Autosomal dominant; ADPKD: Autosomal dominant polycystic kidney disease; PLD: Polycystic liver disease.

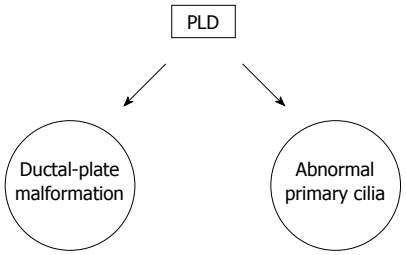

Figure 1 Pathophysiology of polycystic liver disease. PLD: Polycystic liver disease.

ABNORMAL PRIMARY CILIA

Cholangiocytes are the only ciliated cells in the liver. Cilia have mechanosensory capacity and modulate the intracellular levels of cAMP and Ca^{2+} when bent by the flow of bile. They also detect changes in osmolarity and composition of the bile[22-24]. Ciliary defects result in a decreased cytoplasmic level of Ca^{2+} and an increased cytoplasmic level of cAMP[25]. These changes are responsible for the hyperproliferation of cholangiocytes and for the cystogenesis that is a consequence of the altered balance between fluid secretion and absorption in the lumen of the biliary ducts[25].

NATURAL HISTORY AND RISK FACTORS FOR PLD

The natural history of PLD is characterized by a continuous increase in the volume and the number of cysts[11,26,27]. The annual growth of affected livers is in the range of 0.9%-3.2% of the initial hepatic volume[10,28-30]. Both genders are affected; however, women have a higher

Table 2 Risk factors for liver-cyst growth in polycystic liver disease

Risk factors for liver-cyst growth in polycystic liver disease
Advancing patient age
Female gender
Estrogen exposure: multiple pregnancies, OCPs, estrogen replacement therapy
Severity of renal dysfunction and renal cyst volume

OCPs: Oral contraceptive pills.

prevalence. Exposure to estrogen during pregnancies, the use of oral contraceptive pills or estrogen replacement therapy seems to accelerate the progression of the disease[1,27,31]. Other risk factors are the severity of renal dysfunction that is dependent on the volume of the cysts in the kidneys[1]. Table 2 summarizes the known factors that influence the progression of PLD.

CLINICAL PRESENTATION

PLD is asymptomatic in 80% of patients[8,9] and is usually diagnosed incidentally. Women present with massive and symptomatic cystic liver more frequently than men[32]. For 20% of patient, symptoms are typically caused by the compression of organs surrounding the liver, bleeding or infectious complications of the cysts. Compressive symptoms include abdominal distention, early satiety that can lead to decreased oral intake and severe malnutrition, gastro-esophageal reflux, dyspnea, hepatic venous-outflow obstruction (Budd-Chiari syndrome), inferior vena cava syndrome, portal-vein and bile-duct compression. Complications of liver cysts include infections, torsions, rupture and hemorrhage[1,18,33,34] (Table 3). In asymptomatic patients, serum laboratory studies are usually normal. In the presence of symptoms, 47% of patients have elevated serum alkaline phosphatase, 70% have elevated serum levels of gamma glutamil transferase[35-38], 27% have elevated serum levels of aspartate amino transferase and 15% have elevated serum levels of total bilirubin[35,36]. Liver synthetic function is typically preserved despite the presence of innumerable cysts[32] while 45% of patients might have elevated serum tumor marker CA19-9 without proof of malignancy[39]. Other tumor markers such as CA-125, carcinoembryonic antigen, and alpha-fetoprotein may also be elevated but less frequently than CA19-9[40-42].

ASSOCIATED EXTRA-HEPATIC DISEASES

Intracranial arterial aneurysms can affect 6% of patients without a family history of ADPKD and up to 16% of patients with family history of ADPKD. Other common conditions are mitral-valve prolapse and colonic diverticulosis that can be detected in 25% of patients with PLD[1,11,43-45]. Screening for intracranial aneurysm by magnetic resonance angiography (MRA) is recommended only for patients with ADPKD, older than 30 years or for those patients with family history of hemorrhagic strokes or intracranial arterial aneurysms[46]. Screening for intracranial arterial aneurysms is also warranted in cases of a sudden severe headache, or for candidates to liver or kidney transplantation. Screening for mitral-valve prolapse is not recommended unless a cardiac murmur is ascultated during routine clinical examinations[11,47]. Finally, patients with ADPKD may have asymptomatic cysts within other organs, such as the pancreas, spleen, ovaries, and lungs[48]. Pancreatic cysts are the most common with a reported incidence of 9% among ADPKD patients older than 30 years[49-51].

Table 3 Summary of the most frequent symptoms caused by polycystic liver disease

Symptoms due to mass effect	Symptoms due to complications of the cysts
Abdominal distention	Infection
Early satiety	Torsion
Postprandial fullness	Rupture
Gastro-oesophageal reflux	Haemorrhage
Malnutrition	
Dyspnoea	
Hepatic venous-outflow obstruction (Budd-Chiari syndrome)	
Inferior vena cava syndrome	
Portal-vein compression	
Bile-duct compression	

DIAGNOSIS

The most common methods for the diagnosis of PLD are cross sectional imaging studies. Abdominal ultrasound (US) and computerized tomography (CT) are the two most frequent investigations[52,53]. For hepatic cysts, MRI is more sensitive and specific, and it is a valuable test for patients with intravenous contrast allergies or renal dysfunction or when other studies are unable to satisfy the diagnostic needs[54]. Hepatic cysts have radiological characteristics identical to benign developmental cysts. On US, they appear anechoic and well-circumscribed[55]. On CT and MRI, they have non-enhancing, well-circumscribed round walls with hypodense content[55]. On T2-weighted MRI and CT scans, they appear homogenously enhanced spherical lesions[55] (Figure 2B and C). The distinction between isolated PLD and ADPKD relies on the number of renal cysts, age at presentation and family history (Table 4). In adults, younger than 30 years with a positive family history, the diagnosis of ADPKD is established by radiologic evidence of at least two unilateral or bilateral cysts. At least two cysts in each kidney are necessary for the diagnosis of patients between the age of 30 to 59 years, and at least four cysts in each kidney for patients 60 years or older[56]. It is worth noting that at least one third of patients with isolated PLD may also have a few kidney cysts[15,56,57]. It has been proposed that

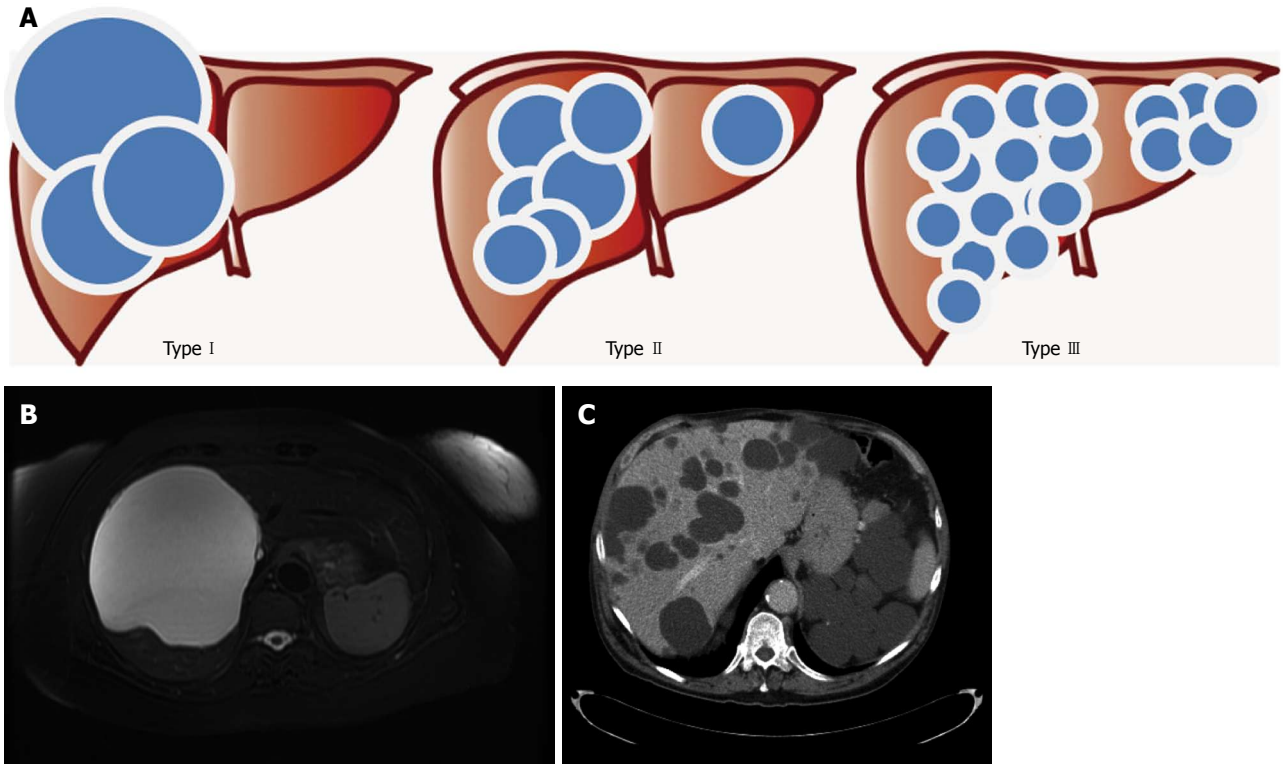

Figure 2 Gigot's classification for polycystic liver diseases. A: Graphical representation; B: Abdominal magnetic resonance imaging of a patient affected by Gigot Ⅰ cystic liver disease; C: Abdominal computerized tomography of a patient affected by Gigot Ⅱ cystic liver disease.

Table 4 The ravine diagnostic criteria for autosomal dominant polycystic kidney disease

Patient's age (yr)	Number of cysts	
	Positive family history	Negative family history
⩽ 30	At least 2 cysts affecting 1 or both kidneys	At least 5 cysts
31-59	At least 2 cysts in each kidney	At least 5 cysts
⩾ 60	At least 4 cysts in each kidney	At least 8 cysts

sporadic cases of PLD should be diagnosed when a patient has more than 15 to 20 cysts and no previous family history[1,18] while four cysts suffice in the presence of a positive familial history[1,18].

INFECTED CYSTS

Hepatic cysts may become infected, and cause life-threatening sepsis[58,59]. Often, infected hepatic cysts are responsible for recurrent episodes of fever without any other signs or symptoms. In these circumstances, the diagnosis can be quite difficult as the accuracy of imaging tests remain low due to the altered anatomy of the liver parenchyma[60]. A promising investigation technique for suspected infected hepatic cysts is In-111 WBC scan[61]. Several other tracers such as 99mTc-diphosphonates, 67Gacitrate, and 111In- or 99mTc-labeled leukocytes have also been used[62]. Although labeled leukocyte imaging is theoretically the test of choice for detecting most infections, it is labor intensive, not always available and involves direct handling of potentially infected blood products. Therefore, considerable effort has been devoted to search for alternatives to this procedure such as the use of 67Gallium scintigraphy and 18F-FDG-positron emission tomography (PET). In recent years, PET has become the most commonly used diagnostic test for the detection of infected renal and hepatic cysts[60,62,63]. However, the accuracy of this technique is still under investigation. The literature on the treatment of infected cysts in PLD patients is very scarce and based only on a few case reports. Most of patients will need parenteral broad spectrum antibiotic therapy with percutaneous drainage of the content of the cyst if their symptoms persists.

CLASSIFICATION

Several clinical classifications have been proposed to grade the severity of PLD.

GIGOT'S CLASSIFICATION

Gigot's classification relies on imaging findings and was designed to identify the best candidates for fenestration of symptomatic cysts[38] (Figure 3): Type Ⅰ: presence of less than 10 large hepatic cysts measuring more than 10 cm in maximum diameter. Type Ⅱ: diffuse involvement of liver parenchyma by multiple cysts with remaining large areas of non-cystic liver parenchyma. Type Ⅲ: presence of diffuse involvement of liver parenchyma by small and medium-sized liver cysts with only a few areas of

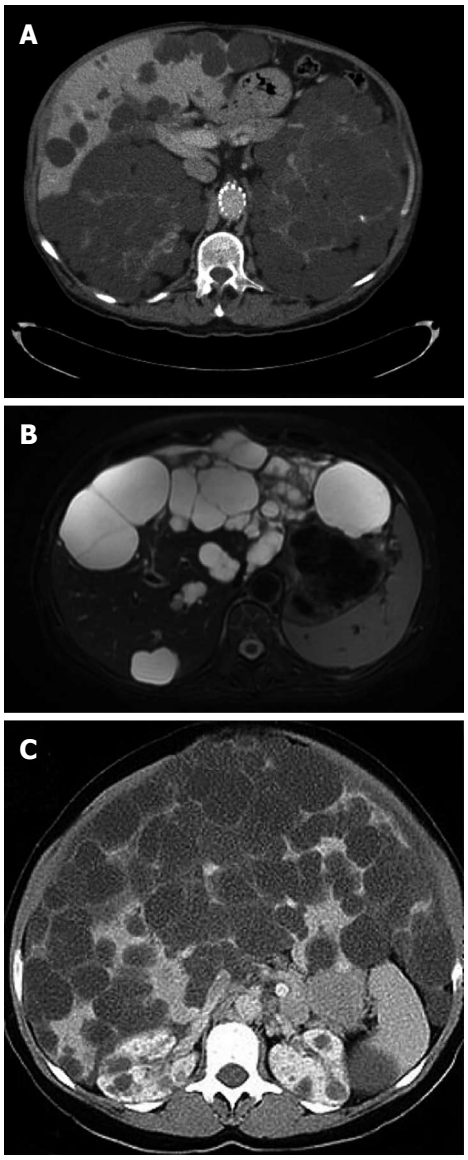

Figure 3 Gigot's classification relies on imaging findings and was designed to identify the best candidates for fenestration of symptomatic cysts. A: Intravenous contrast enhanced computerized tomography (CT) of a patient affected by polycystic liver and renal disease. The cysts appears hypoattenuating with smooth and regular walls; B: T2 magnetic resonance imaging of a patient with multiple hepatic cysts. The cystic fluid appears bright on T2 images; C: Abdominal CT of a patient affected by Gigot Ⅲ cystic liver disease.

normal liver parenchyma.

QUIAN'S CLASSIFICATION

Qian's classification has been used in the context of familial screening and relies on the number of cysts and the presence of symptomatic hepatomegaly[18]: (1) grade 0 - 0 cysts; (2) grade 1 - 1 to 10 cysts; (3) grade 2 - 11 to 20 cysts; (4) grade 3 - more than 20 cysts; and (5) grade 4 - more than 20 cysts and symptomatic hepatomegaly.

SCHNELLDORFER'S CLASSIFICATION

Schnelldorfer's classification aims at differentiating patients who could benefit from resection or transplantation as summarized in Table 5[64].

TREATMENT

Most patients with PLD are asymptomatic and do not require any intervention[6]. However, symptomatic PLD patients might require treatment when they experience severe dysfunction of organs around the liver due to the increased hepatic volume or when one or more cysts get torted, infected or develop intra-cystic hemorrhages (Table 6).

AVOIDANCE OF EXPOSURE TO ESTROGENS

Observational and experimental studies have shown that PLD may worsen under the influence of estrogen during pregnancy or when patients are prescribed estrogen replacement therapy[1,27,31]. Estrogen can increase both the number of liver cysts and their volume, therefore, hormonal therapy should be stopped in most symptomatic patients when appropriate[27].

NON-SURGICAL TREATMENTS

Medical management may be valuable in symptomatic patients with Gigot's type Ⅱ/Ⅲ.

SOMATOSTATIN ANALOGUES

Somatostatin analogues are inhibitors of cAMP and they reduce the secretion of fluid and the proliferation of many cell types, including cholangiocytes[65-69]. They also suppress the expression of insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), and other cytogenetic growth factors[70]. In addition, somatostatin analogues inhibit the downstream signaling of these receptors[70]. Two randomized controlled trials have recently demonstrated that after 6 to 12 mo, treatment with lanreotide, a long-acting somatostatin analogue, was associated with a significant reduction of liver volume in patients with PLD compared with placebo[28,29]. However, the average hepatic volume reduction was only 3% to 5%. The severity of abdominal symptoms was also not significantly improved[28]. Currently, somatostatin analogues are indicated only for a selected group of patients with symptomatic PLD in whom the risks for surgical intervention are not justified, or in whom the surgical intervention is technically challenging.

MAMMALIAN TARGET OF RAPAMYCIN INHIBITORS

Mammalian target of rapamycin (m-TOR) inhibitors have immunosuppressive and antiproliferative effects[71]. Sirolimus and Everolimus were studied in Phase-Ⅱ prospective randomised control trials. None of the two drugs showed substantial therapeutic effects both in hu-

Table 5 Summary of Schnelldorfer's classification that aims at differentiating patients who could benefit from resection or transplantation

	Type A	Type B	Type C	Type D
Symptoms	Absent or mild	Moderate or severe	Severe (or moderate)	Severe (or moderate)
Cyst characteristics	Any	Limited No. large cysts	Any	Any
Areas of relative normal liver parenchyma	Any	⩾ 2 sectors	⩾ 1 sector	< 1 sector
Presence of portal vein or hepatic vein occlusion in the preserved hepatic sectors	Any	Absent	Absent	Present
Recommended therapy	Observation or medical therapy	Cyst fenestration	Partial hepatectomy with possible fenestration of remnant cysts	Liver Transplantation

Table 6 Summary of treatment options for polycystic liver disease

Treatment approach	Treatment type
Nonsurgical	Medical
	Somatostatin analogues
	mTOR inhibitors
	Interventional radiology:
	Arterial embolization
	Percutaneous sclerotherapy
Surgical	Fenestration
	Hepatic resection with fenestration
	Liver transplantation

OCP's: Oral contraceptive pills; mTOR: Mammalian target of rapamycin.

mans[72-74] and in animal models[75]. Clinical prospective data o the effect of m-TOR inhibitors are currently not available, and this class of medications should not be recommended outside clinical trials.

INTERVENTIONAL RADIOLOGY: ARTERIAL EMBOLIZATION

Trans-catheter arterial embolization has been used since the early 2000s[76]. Hepatic artery branches supplying the hepatic segments replaced by the cysts are targeted by using microcoils or polyvinyl alcohol particles measuring 150-250 μm in diameter[76,77]. For patients with advanced PLD and multilobal disease, trans-catheter arterial embolization can be technically demanding. The largest series of patients treated with this modality included 30 patients who had a significant reduction of the volume of their cysts (6.667 ± 2.978 cm^3 down to 4.625 ± 2.299 cm^3), whereas the volume of the unaffected hepatic parenchyma increased[76]. After several months, patients reported improvement of their symptoms and no major complications except for occasional post-embolization syndrome[76,77].

PERCUTANEOUS SCLEROTHERAPY

This technique requires radiologically guided percutaneous aspiration of the content of the cysts followed by the injection of a sclerosing agent that inhibit the reaccumulation of fluid by damaging the epithelial lining the cystS[78,79]. Symptomatic patients with one to five large dominant cysts (Gigot's type Ⅰ) are suitable for percutaneous sclerotherapy. Most commonly, cysts with a diameter larger than 5 cm are candidates for this treatment[10]. Puncturing of the cyst can be done with a 5 or 7 French catheter[80] and sclerosing agents commonly used include ethanol, ethanolamine oleate, minocycline and tetracycline. Although a single session is often sufficient, some patients require more than one[81]. Aspiration with sclerotherapy has an excellent safety profile, although severe abdominal pain can be caused by peritoneal irritation due to spillage of the sclerosing agent[10]. The majority of patients who undergo percutaneous sclerotherapy has improved symptoms in the immediate period following the procedure[10], but only 20% will have partial, or full regression of their disease[10].

SURGERY

Patient and treatment selection remain a clinical challenge. There is no consensus on selection criteria for surgery, the optimal timing, and technique. Current surgical options include fenestration, partial liver resection and OLT. Fenestration and partial liver resection are options for Gigot's type Ⅰ and Ⅱ patients. For Gigot's type Ⅲ disease, fenestration and partial liver resection are often ineffective, and OLT should be considered as it is the only curative treatment. In general, several factors have to be considered before any surgical intervention is recommended: (1) The degree of cystic burden; (2) The distribution of the cysts; and (3) The proximity of the cysts to the main biliary ducts and portal and hepatic vein branches.

SURGICAL PEARLS

In Gigot's type Ⅰ or Ⅱ, symptoms might not be related to the size of the entire liver but to the size of one or two large cysts. These patients can be treated similarly to those with simple cysts. Some hepatic segments such as V and VI are frequently spared and, therefore, surgical resection can be performed if the spared liver parenchyma is thought to be sufficient. Frequently, the right hepatic veins are compressed by cysts causing the formation of collateral circulation between the right and the middle hepatic veins that can be responsible for intraoperative bleeding during the parenchymal transaction.

Table 7 Summary of largest series published on the surgical techniques used for cystic fenestration of symptomatic polycystic liver disease

Ref.	No. of patients	Technique	Outcome	Complications	Follow-up (mo)
van Erpecum *et al*[35]	15	Open fenestration	0% symptom recurrence	One mortality	Mean of 48
Kabbej *et al*[37]	13	Lap fenestration	72% symptom recurrence	54% morbidity	Mean follow-up 26
Gigot *et al*[38]	10	Open fenestration	11% symptom recurrence	60% morbidity	73 mean follow-up
van Keimpema *et al*[82]	12	Lap fenestration	Reduction in liver volume by 12.5%	Bile leak, vena cava occlusion and sepsis	-
Pirenne *et al*[92]	4	Lap fenestration	100% symptom relief	50% cyst recurrence	-
Liska *et al*[95]	7	Lap fenestration plus open	-	No mortality	Mean 41
Bai *et al*[96]	10	Lap fenestration	Symptom and cyst recurrence in 20%	3 patients with minor complications. No mortality	Mean of 57
Palanivelu *et al*[97]	4	Lap fenestration	100% cyst recurrence	-	-
Garcea *et al*[98]	6	Lap/Open fenestration	16.7% symptom recurrence, 33.3% cyst recurrence	50% morbidity	5-36
Neri *et al*[99]	3	Lap fenestration	100% symptom relief	50% morbidity	-
Kornprat *et al*[100]	8	Lap fenestration	0% symptom recurrence	-	-
Robinson *et al*[101]	11	Lap fenestration	54.5% symptom recurrence	-	-
Fiamingo *et al*[102]	6	Lap fenestration	30% symptom recurrence	50% morbidity	1-64
Tocchi *et al*[103]	18	Lap/open fenestration	-	-	-
Koperna *et al*[104]	39	Open fenestration (n = 34); Lap (n = 5)	21% symptom recurrence	-	75 mean follow-up
Morino *et al*[105]	7	Lap fenestration	40% symptom recurrence	44% morbidity rate	-
Farges *et al*[106]	13	Open fenestration	23% symptom recurrence	69% morbidity	84 follow-up
Ueno *et al*[118]	13	Open fenestration (n = 6); Lap (n = 13)	71% symptom recurrence	30% morbidity	37 mean follow-up

Lap: Laparoscopic.

FENESTRATION

Fenestration is a surgical technique that combines aspiration and surgical unroofing of the cyst. It has the advantage that multiple cysts can be treated in one session[48,82]. Fenestration is effective in symptomatic patients with Gigot's type I and II disease[83]. Patients with superficial and a limited number of large cysts are the best candidates for this procedure[48]. Fenestration may be achieved by laparotomy or laparoscopy[48]. Patients with the majority of their cysts located in the right posterior segments (VI, VII), or at the dome of the liver (segment VIII) may be better candidates for open fenestration because these cysts are difficult to be visualized and fenestrated by laparoscopic approach[48]. Published series describing open and laparoscopic fenestration are summarized in Table 7. Immediate symptom relief is achieved in 92% of the patients, whereas up to 25% experience recurrence of the cysts or symptoms[10]. Complication rate after fenestration is in the range of 23% while mortality is about 2%[10]. Complications include ascites, pleural effusion, hemorrhage and bile leakage[84]. Factors that predict failure of fenestration are previous abdominal procedures, deep-seated cysts, incomplete unroofing, cysts in segments VII-VIII, and the presence of diffuse PLD[10].

HEPATIC RESECTION WITH FENESTRATION

Hepatic resection is usually reserved for highly symptomatic patients who are incapacitated by their disease due to the massive expansion of their livers (Gigot's type II and III)[38]. In these circumstances fenestration alone is rarely successful because the liver parenchyma is rigid and it does not collapse[10]. Symptom relief is achieved in 86% of cases although cyst recurrence is expected in one third of patients[10]. Overall, most of the patients have an improvement in their quality of life and functional status[36]. The morbidity rate associated with this procedure can be up to 50% and includes ascites, pleural effusions, biliary leakage, and hemorrhage[10]. One of the reasons for these complications is the fact that there is a significant distorsion of the intra-hepatic vasculature and biliary tree which makes these procedures technically very challenging. Mortality rate is around 3%[10]. As subsequent adhesions may complicate future OLT, this surgical treatment is usually preserved for patients with massive hepatomegaly for which OLT is not an option[85,86]. Published series describing hepatic resection with/without fenestration for symptomatic PLD are summarized in Table 8.

LIVER TRANSPLANTATION

OLT is the only curative treatment for patients with severe PLD[87]. It is indicated in those patients with disabling symptoms that lead to decreased performance status and quality of life[10]. Patients with PLD usually have normal liver function and the current organ allocation system based on the Model for End-Stage Liver Disease (MELD) is often unable to assist this group of patients. For these patients, MELD exception criteria are needed[88,89]. Because of the shortness of available grafts, the need for life-long immunosuppression and the perioperative risks, OLT is indicated only for symptomatic patients

Table 8 Summary of largest series published on the surgical techniques used for cystic fenestration and resection of symptomatic polycystic liver disease

Ref.	No.	Technique	Outcome	Complications	Follow-up (mo)
Que *et al*[36]	31	Fenestration and resection	3% symptom recurrence	3% mortality, 58% morbidity	Mean of 28
Schnelldorfer *et al*[64]	124	Fenestration and resection	93% symptom relief, 72.6% recurrent cyst formation	72.6% morbidity, 3.2% mortality	Mean of 48
Kornprat *et al*[100]	9	Fenestration and resection	100% symptom relief, 11% recurrence	33.35% morbidity	24-98
Koperna *et al*[104]	5	Fenestration and resection	0% symptom recurrence	-	-
Li *et al*[107]	21	Fenestration and resection	14.3% cyst recurrence	76.2% cyst morbidity, 0% mortality	10-155
Gamblin *et al*[108]	51	Fenestration and resection	3.9% symptom recurrence	17.6% morbidity, no mortality	1-49
Yang *et al*[109]	7	Fenestration and resection	100% symptom recurrence	100% morbidity, no mortality	Mean of 20
Vons *et al*[110]	12	Resection	17% symptom recurrence	8% mortality, 83% morbidity	Mean of 34
Soravia *et al*[111]	10	Fenestration and resection	33% symptom recurrence	10% mortality, 20% morbidity	Mean of 69
Henne-Bruns *et al*[112]	8	Fenestration and resection	50% symptom recurrence	No mortality, 38% morbidity	Mean of 15
Vauthey *et al*[113]	5	Fenestration and resection	0% symptom recurrence	0% mortality, 100% morbidity	Mean of 14
Sanchez *et al*[114]	9	Resection	100% symptom relief, 100% recurrence	0% mortality	Mean of 35
Newman *et al*[115]	9	Fenestration and resection	88.9% symptom relief, 0% recurrence	11.1% mortality, 55.6% morbidity	2-44
Iwatsuki *et al*[116]	9	Resection	44.4% symptom relief, 44.4% recurrence	0% mortality, 33.3% morbidity	12-180

Table 9 Summary of largest series published on the outcomes of patients undergoing liver transplantation for symptomatic polycystic liver disease

Ref.	No. of patients	Previous surgery	Combined liver and kidney transplantation	Morbidity	Mortality	Follow-up (mo)	Re-transplantation
Pirenne *et al*[92]	16	25%	6%	38%	13%	Range 18-120	0%
Taner *et al*[117]	13	-	54%	85%	31%	-	0%
Ueno *et al*[118]	14	-	36%	64%	21%	-	0%
Ueda *et al*[119]	3	-	0	33%	0%	Mean of 32	0%
Gustafsson *et al*[120]	7	57%	43%	57%	43%	Mean of 4	0%
Swenson *et al*[121]	9	44%	33%	44%	11%	Mean of 26	11%
Lang *et al*[122]	17	35%	47%	47%	29%	Mean of 12	12%
Washburn *et al*[123]	5	90%	20%	0%	20%	Mean of 38	0%
Starzl *et al*[124]	4	0%	25%	0%	50%	Mean of 38	0%

Table 10 Suggested management strategies based on Gigot's classification

Gigot's Ⅰ	Gigot's Ⅱ - Ⅲ
Percutaneous sclerotherapy	Hepatic resection with fenestration if feasible
Fenestration	Liver transplantation

with Gigot's type Ⅱ and Ⅲ disease[12,48,90]. For patients undergoing OLT for PLD, perioperative morbidity is 40%-50%, whereas overall mortality is 10%-17%[10]. In 3% of patients, retransplantation is required[10] and combined renal and liver transplantation are necessary in 42% of patients[91,92]. Expected survival at 1- and 5-year are 93% and 92% for patients undergoing OLT alone while for patients who undergo combined liver and kidney transplant are 86% and 80% respectively[10]. Published series reporting the outcomes of OLT for symptomatic PLD are summarized in Table 9.

HEPATIC RESECTION VS LIVER TRANSPLANTATION

The clinical decision between performing a hepatic resection with or without cyst fenestration[93] and referring the patient for OLT can be extremely difficult (Table 10). Hepatic resection with cyst fenestration implies leaving residual hepatic cysts that will eventually progress[94]. However, hepatic resection is associated with a lower risk of perioperative morbidity and mortality. OLT provides the only option for the cure of these patients but requires lifelong immunosuppression and has higher perioperative risks. Both resection and OLT are technically demanding, and peri-operative care can be complex. The risks and the benefits of each of the possible treatment options have to be carefully evaluated and put in the contest of the clinical presentation and condition of each patient. Referral to a tertiary center with an experienced team of surgeons, hepatologists, and nephrologists is strongly recommended.

CONCLUSION

For patients with PLD, patients' selection, timing and choice of treatments can be very challenging even for experienced physicians. For symptomatic patients, treatment strategies should be based on the degree and progression of their symptoms and the severity of other medical conditions. Symptomatic patients with large cysts or limited hepatic involvement might benefit from fenestration or sclerotherapy. Hepatic resection with or without fenestra-

tion should be favored in patients with diffuse involvement of the liver but with sufficient spared parenchyma. Finally, in the patient with diffuse disease, OLT is a valid option and should be pursued as primary therapy prior to the development of debilitating disease such as malnutrition and liver dysfunction that can significantly increase the risks of perioperative adverse events.

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REVIEW

Overlap of functional heartburn and gastroesophageal reflux disease with irritable bowel syndrome

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Abstract

Several studies indicate a significant degree of overlap between irritable bowel syndrome (IBS) and gastroesophageal reflux disease (GERD). Likewise, both functional heartburn (FH) and IBS are functional digestive disorders that may occur in the same patients. However, data establishing a solid link between FH and IBS are lacking, mainly because the clinical definition of FH has undergone substantial changes over the years. The available literature on the overlap between GERD or FH and IBS highlights considerable heterogeneity in terms of the criteria and diagnostic procedures used to assess heartburn and IBS. In particular, several epidemiological studies included patients with concomitant IBS and GERD without any attempt to distinguish FH (as defined by the Rome Ⅲ criteria) from GERD *via* pathophysiological investigations. Independent of these critical issues, there is preliminary evidence supporting a significant degree of FH-IBS overlap. This underscores the need for studies based on updated diagnostic criteria and accurate pathophysiological classifications, particularly to distinguish FH from GERD. This distinction would represent an essential starting point to achieving a better understanding of pathophysiology in the subclasses of patients with GERD and FH and properly assessing the different degrees of overlap between IBS and the subcategories of heartburn.The present review article intends to appraise and critically discuss current evidence supporting a possible concomitance of GERD or FH with IBS in the same patients and to highlight the pathophysiological relationships between these disorders.

Key words: Functional gastrointestinal disorders; Gastroesophageal reflux disease/Gastro-oesophageal reflux disease; Irritable bowel syndrome; Acidity (esophageal); Hypersensitivity

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INTRODUCTION

Gastroesophageal reflux disease (GERD) and irritable bowel syndrome (IBS) are gastrointestinal disorders that affect a large portion of the general population and have a relevant impact on quality of life and health care costs. Although these disturbances affect different regions of the digestive tract, it has been noted that they may occur in the same patient. In addition, recent studies have shown a concomitance between functional heartburn

(FH) and IBS. This finding is not completely unexpected because FH and IBS are both functional digestive disorders (FDDs), and the possibility of an overlap among different FDDs has been largely acknowledged[1]. Indeed, there is mounting evidence that FDDs consist of a number of heterogeneous syndromes characterized by various gastrointestinal symptoms with no evident organic cause found upon clinical investigation[2].

Based on the above considerations, the hypothesis of an association between FH and IBS deserves careful attention and investigation. However, data establishing a solid link between FH and IBS are lacking, most likely because the disorders' clinical definitions have undergone to significant variations over the years, and their pathophysiology remains poorly understood.

The present article intends to provide a review of current evidence supporting a possible clinical and pathophysiological relationship between GERD/FH and IBS.

DEFINITIONS

To properly address the relationship between GERD/FH and IBS, it is important to preliminarily clarify some definitions of GERD, as patients affected by FH have been often included in this category in both past and recent clinical investigations.

GERD

GERD develops when the reflux of gastric contents into the esophagus leads to troublesome symptoms, with or without mucosal damage and/or complications[3]. A subcategory of GERD patients that displays reflux-related symptoms in the absence of erosive esophagitis at endoscopy is considered to have non-erosive reflux disease (NERD)[3]. Pathophysiological studies conducted *via* pH monitoring and, more recently, impedance-pH monitoring (MII-pH) have demonstrated that there are two main types of NERD patients: those with abnormal acid reflux and those with physiological acid exposure time (AET). In the latter group, patients showing a close temporal relationship between symptoms and acid or non-acid reflux episodes have been defined as having a "hypersensitive esophagus" and should be considered within the spectrum of GERD[4,5]. When the association between symptoms and physiological reflux is lacking, patients can be classified as having FH, which is defined in the next section.

FH

The Rome Ⅱ criteria for functional esophageal disorders defined FH as an episodic retrosternal burning in the absence of pathological gastroesophageal reflux, pathology-based motility disorders, or structural alterations[6]. In 2006, the Rome Ⅲ committee modified the definition of FH as the occurrence of chronic retrosternal burning in the absence of either GERD or histopathology-based esophageal motility disorders. In particular, according to Rome Ⅲ criteria, heartburn should be reported as having persisted over the previous 3 mo, with a symptom onset dating to at least 6 mo before the diagnosis[7]. To exclude GERD, patients must undergo upper digestive endoscopy; in the absence of esophagitis, ambulatory pH monitoring should also be performed[4]. A lack of correspondence between symptoms and reflux episodes, together with normal acid exposure in the distal esophagus, would suggest a diagnosis of FH. Such a diagnosis could be further substantiated by the outcome of a therapeutic trial with a proton pump inhibitor (PPI); although it is not specific, an unsatisfactory response to acid inhibition is likely to have a negative predictive value in support of GERD[8].

A recent study suggested that, to be diagnosed with FH, patients should have a normal upper endoscopy, a normal AET in the distal esophagus and a negative symptom association with both acid and non-acid reflux[5].

The evaluation of the latter condition is possible only with MII-pH monitoring, which is able to recognize both acid and non-acid reflux. However, it must be considered that, to date, the exact role of non-acid reflux in the pathophysiology of symptoms in untreated GERD patients has been minimally evaluated. Therefore, the findings reported by Savarino *et al*[5] should be viewed as preliminary in nature and should be substantiated by further studies before undergoing a critical assessment by consensus committees.

IBS

According to the Rome Ⅲ criteria, IBS is a functional bowel disorder in which recurrent abdominal pain or discomfort is associated with defecation and/or changes in bowel habits. In particular, abdominal pain or discomfort is associated with two or more of the following characteristics: improvement with defecation and onset associated with a change in the frequency and/or form of stool. The predominant stool pattern allows the classification of IBS into four clinical variants: with constipation; with diarrhea; mixed; and unsubtyped[9].

GERD/FH AND IBS OVERLAP

To date, several studies have reported a certain degree of overlap between GERD and IBS that cannot be explained solely by chance[10-13]. By contrast, epidemiological data regarding the possible concomitance of FH and IBS in the same patient are lacking.

In the last two decades, the assessment of the epidemiological and clinical features of IBS has gained considerable attention. At present, the overall prevalence of IBS ranges from 10% to 20% of adults and adolescents, and it predominantly affects young (20-45 years old) females[14,15]. Population-based studies suggest that GERD, defined by at least weekly heartburn and/or regurgitation, is a common condition, with a prevalence of 10%-20% in Western populations[16]. Several studies have shown that up to 70% of patients complaining of heartburn have NERD; 30%-50% of NERD patients display nor-

mal 24-h esophageal pH monitoring[17], and approximately 60% of these patients show a negative relationship between symptoms and acid reflux events[4]. More recent studies conducted with MII-pH in NERD patients suggest an FH prevalence ranging from 19% to 26%[5]. Very little is currently known about gender prevalence among patients with FH, although the condition seems to be more common in women[18].

The identification of a clinical overlap between FH and IBS is complicated by the fact that most studies have usually evaluated the concomitance of IBS and heartburn, irrespective of whether the latter was related to GERD or FH. In particular, most data have been collected *via* epidemiological studies conducted using validated questionnaires and endoscopy, without any pathophysiological attempt to discriminate GERD patients from FH patients. In this context, we were interested in performing an in-depth analysis of the overlap between GERD/FH and IBS by conducting a search of the available literature.

Literature search

We identified the published studies to include in our review *via* an electronic search of three bibliographical databases: PubMed (1966-2011), EMBASE (1980-2011) and the Cochrane Library (2000-2011). Only studies that were designed as randomized-controlled, cross-sectional and case-control were included in our analysis. The search was performed by two investigators using the string "(reflux OR heartburn OR GERD OR GORD OR gastroesophageal reflux OR PPI OR 24-h pH) AND (IBS)". A restriction was placed to collect articles in English only. The initial search yielded 371 titles of studies that were published as either full text papers or abstracts of scientific meetings, and all of the studies were screened by all authors to determine their eligibility. Based on our inclusion criteria, we selected 45 studies, which were used for an in-depth analysis of the prevalence of GERD/FH in patients with IBS and vice versa. In addition, the criteria and diagnostic procedures used to assess the presence of heartburn and IBS were recorded.

Prevalence of GERD/FH in patients with IBS

Twenty-three studies evaluated the prevalence of GERD/FH in subjects with a previous diagnosis of IBS[10,12,19-39]. The details are shown in Table 1. The overall mean prevalence of GERD was 37.5%, although there was remarkable variability, with values ranging from 11% to 79%. Five studies assessed IBS according to the Manning criteria, 4 studies according to the Rome I criteria, 8 studies according to the Rome Ⅱ criteria, and 6 studies according to the Rome Ⅲ criteria. In 18 studies, IBS was diagnosed *via* a symptom questionnaire; in 4 studies, organic diseases were excluded with imaging techniques and laboratory tests; in 1 study, only laboratory tests were performed. In comparison, GERD was diagnosed *via* a symptom questionnaire in 18 studies and a symptom questionnaire combined with upper endoscopy in 3 studies. In 2 studies, pathophysiological evaluations *via* esophageal manometry and pH-metry/MII-pH were performed in addition to the symptom questionnaire and upper endoscopy[20,39]. Overall, in patients with IBS, NERD was slightly more prevalent (42%) than erosive reflux disease (ERD, 38%). One study conducted in accordance with Rome Ⅲ criteria estimated an FH prevalence of 59% among patients with IBS[39].

Prevalence of IBS in patients with GERD/FH

Thirty-two articles investigated the prevalence of IBS in subjects with a previous diagnosis of GERD/FH[10,12,23,25,26,31,32,35,36,38-60]. The details are shown in Table 2. In GERD patients, the overall mean prevalence of IBS was 36%, although there was considerable variability, as shown by values ranging from 8% to 71%. In 3 studies, IBS was diagnosed according to the Manning criteria (mean prevalence: 34.4%); in 8 studies, it was diagnosed according to the Rome I criteria (mean prevalence: 41.4%); in 10 studies, it was diagnosed according to the Rome Ⅱ criteria (mean prevalence: 38.1%); in 8 studies, it was diagnosed according to the Rome Ⅲ criteria (mean prevalence: 31.9%); in 3 studies according to the ReQuest criteria (mean prevalence: 37.3%). In all studies, IBS was diagnosed *via* a symptom questionnaire. However, in one study, hematological and stool examinations were also performed to exclude organic diseases[26]. In comparison, GERD was diagnosed *via* a symptom questionnaire in 18 studies and *via* a symptom questionnaire combined with upper endoscopy in 7 studies. In 7 studies, esophageal pathophysiological studies (*i.e.*, manometry and pH-metry) were performed in addition to the symptom questionnaire and upper endoscopy. Overall, IBS was more prevalent in patients with NERD (41%) than in those with ERD (23.9%). Two studies, which evaluated FH in accordance with the Rome Ⅲ criteria, estimated prevalences of 39%[56] and 61.4% for IBS[39]. In the first study, heartburn was investigated *via* pH-metry, while the latter used MII-pH testing.

Discussion

Large population-based studies have used validated questionnaires to investigate a possible association between GERD and IBS and have suggested that GERD can affect a considerable proportion of patients with IBS[22,27,28] or vice versa[43,49]. However, few studies specifically address the issue of overlap between FH and IBS, mainly because the definition of FH has varied substantially throughout the years. Indeed, the definition of FH has been greatly modified from the Rome Ⅱ criteria (in which the definition of FH included all NERD patients with negative pH-metry) to the Rome Ⅲ criteria (in which FH is defined as a functional esophageal disorder unrelated to GERD and characterized by negative pH-metry, the lack of a relationship between symptoms and reflux events, and the lack of symptom improvement after a trial of PPI therapy).

Notably, most of the available data on the association between IBS and GERD were collected in the context

Table 1 Prevalence of gastroesophageal reflux disease/functional heartburn in irritable bowel syndrome patients

IBS patients (*n*)	IBS criteria	Diagnostic investigations of IBS	GERD prevalence	FH prevalence	Diagnostic investigations of heartburn	Ref.
101	Manning	S, HE, Sg, BE, BT, UE, SBB, BC, LE	25%	Not evaluated	SQ	Svedlund *et al*[19]
25	Manning	S, Sg, SC, HE, BE	28% (daily) 52% (weekly)	Not evaluated	S, UE, OM, pH (wireless)	Smart *et al*[20]
100	Manning	S, LE, HE, BE	30%	Not evaluated	SQ	Whorwell *et al*[21]
350	Modified manning	SQ	79%	Not evaluated	SQ	Jones *et al*[22]
546	Modified manning	Postal SQ	46.5%	Not evaluated	Postal SQ	Kennedy *et al*[23]1
146	Rome I	S, PE, AU, HE, UE or BE (patients older than 50 yr)	28%	Not evaluated	S, UE	Stanghellini *et al*[24]
68	Rome I	SQ	3%	Not evaluated	SQ	Hu *et al*[25]1
68	Rome I	Phone SQ	11%	Not evaluated	Phone SQ	Cheung *et al*[12]1
52	Rome I	S, SC, HE	38% (ERD) 42% (NERD)	Not evaluated	S, UE	Camacho *et al*[26]1
76 (IBS-C) 45 (IBS-D)	Rome II	SQ	32.9% 40.9%	Not evaluated	SQ	Talley *et al*[27]
3880	Rome II Rome I Manning	Phone SQ	21%	Not evaluated	Phone SQ	Hungin *et al*[28]
662	Rome II	SQ	25%	Not evaluated	SQ	Si *et al*[29]
517	Rome II	SQ	40%	Not evaluated	SQ	Balboa *et al*[30]
95	Rome II	SQ	21%	Not evaluated	SQ	Lee *et al*[31]1
40	Rome II	SQ	20%	Not evaluated	SQ	Hori *et al*[32]1
164	Rome II	SQ	43%	Not evaluated	SQ	Johansson *et al*[33]
113	Rome II	SQ	49.6%	Not evaluated	SQ	Schmulson *et al*[34]
252	Rome III	Postal SQ	32.9%	Not evaluated	Postal SQ	Jung *et al*[10]1
1419	Rome III Rome II	SQ	63.6%	Not evaluated	S, UE	Yarandi *et al*[35]1
381	Rome III	SQ	16%	Not evaluated	SQ	Kaji *et al*[36]1
1336 (in 1996) 799 (in 2006)	Rome III Rome II Manning	Postal SQ	60.5%-71.9%	Not evaluated	Postal SQ	Olafsdottir *et al*[37]
381	Rome III	SQ	16%	Not evaluated	SQ	Fujiwara *et al*[38]1
46	Rome III	SQ	41.3%	59%	S, UE, OM22, MII-pH	Martinucci *et al*[39]1,2

[1]Articles listed in both Tables 1 and 2; [2]Abstract only (publication type). GERD: Gastroesophageal reflux disease; FH: Functional heartburn; IBS: Irritable bowel syndrome; S: Symptoms; SQ: Symptom questionnaire; PE: Physical examination; HE: Hematological examinations; BE: Barium enema; BC: Bacteriological culture; SC: Stool culture; BT: Lactose/lactulose breath test; AU: Abdominal ultrasonography; UE: Upper endoscopy; LE: Lower endoscopy; SBB: Small-bowel biopsies; Sg: Sigmoidoscopy; OM: Esophageal manometry; pH: pH-metry; MII-pH: pH impedance monitoring; ERD: Erosive reflux disease; NERD: Nonerosive reflux disease.

of epidemiological studies, which were conducted on patients with heartburn using validated questionnaires and upper endoscopy without the use of any reliable pathophysiological investigation to discriminate FH (according to the Rome Ⅲ criteria) from GERD.

As mentioned above, only two studies have evaluated the concomitance of FH and IBS. Lee *et al*[56] examined 95 patients with heartburn by endoscopy, pH-metry, PPI test, and psychological characteristics. The patients were classified using the Rome Ⅲ criteria; therefore, FH was diagnosed based on physiological AET, a negative association between symptoms and reflux, and a negative PPI test in patients without erosive esophagitis. A higher prevalence of IBS was recorded in FH patients (39%) than in ERD (17%) or NERD (23%) patients. Furthermore, anxiety was more prevalent in FH patients than in NERD patients. Recently, we examined 92 patients with heartburn (without esophageal mucosal breaks found upon upper endoscopy) *via* pH-MII to assess, in accordance with Rome Ⅲ criteria, the prevalence of NERD subgroups and FH in two groups of patients: those with and those without IBS. For each subject, we evaluated the AET, number of reflux episodes, correlation between symptoms and refluxes, and subjective response to PPI therapy. FH was found in 59% (27/46) of the patients with IBS, compared with 37% (17/46) of the patients without IBS ($P < 0.05$), indicating a higher prevalence of FH in IBS patients. In comparison, IBS was found in 39.6% (19/48) of the patients with NERD and in 61.4% (27/44) of the patients with FH, suggesting that in IBS patients, FH was more common than NERD was[39]. Although data from these two pioneering studies are not sufficient to support the concept that FH and IBS can occur in the same patient, they underscore the need for future investigations based on updated diagnostic criteria.

PATHOPHYSIOLOGICAL SIMILARITIES IN GERD, FH AND IBS

Previous studies dealing with the overlap between GERD

Table 2 Prevalence of irritable bowel syndrome in gastroesophageal reflux disease/functional heartburn patients

GERD patients (*n*)	FH patients (*n*)	Diagnostic investigations of heartburn	IBS prevalence	IBS criteria	Diagnostic investigations of IBS	Authors
910	Not evaluated	Postal SQ	19%	Manning	Postal SQ	Kennedy *et al*[23]1
80	Not evaluated	SQ	36.7%-45.1%	Manning	SQ	Chey *et al*[40]2
34 (ERD) 67 (NERD)	Not evaluated	S, UE	36% (in ERD) 35% (in NERD)	Manning	SQ	Nojkov *et al*[41]
643	Not evaluated	SQ	42%	Rome I	SQ	Locke *et al*[42]
35	Not evaluated	SQ	71%	Rome I	SQ	Pimentel *et al*[43]
79	Not evaluated	SQ	3%	Rome I	SQ	Hu *et al*[25]1
457	Excluded	S, UE, OM, pH	49%	Rome I	SQ	Zimmerman *et al*[44]
79	Not evaluated	Phone SQ	13%	Rome I	Phone SQ	Cheung *et al*[12]1
326 (NERD)	Excluded	S, UE, pH	48.5%	Rome I	SQ	Hershcovici *et al*[45]
326 (NERD)	Excluded	S, UE, pH	49%	Rome I	SQ	Zimmerman *et al*[46]
41 (ERD) 45 (NERD)	Not evaluated	S, UE	48.7% (in ERD) 48.8% (in NERD)	Rome I	S, SC, HE	Camacho *et al*[26]1
3318	Not evaluated	SQ	36.7%-45.1%	Rome II	SQ	Bueno *et al*[47]2
102	Excluded	S, UE, OM, pH	32.4%	Rome II	SQ	Raftopoulos *et al*[48]
3318	Not evaluated	SQ	27%	Rome II	SQ	Guillemot *et al*[49]
263	Not evaluated	S, pH	35%	Rome II	SQ	De Vries *et al*[50]
111 (ERD) 113 (NERD)	Excluded	S, UE, OM, pH.	15.3% (in ERD) 44.2% (in NERD)	Rome II	SQ	Wu *et al*[51]
238	Not evaluated	SQ	60.9%	Rome II	SQ	Nasseri-Moghaddam *et al*[52]
67	Not evaluated	SQ	27%	Rome II	SQ	Lee *et al*[31]1
16	Not evaluated	SQ	50%	Rome II	SQ	Hori *et al*[32]1
92	Not evaluated	SQ	62%	Rome II	SQ	Rey *et al*[53]
102 (ERD) 163 (NERD)	Excluded	S, UE, OM, pH	20.6% (in ERD) 39.9% (in NERD)	Rome II	SQ	Wu *et al*[54]
411	Not evaluated	Postal SQ	20.2%	Rome III	Postal SQ	Jung *et al*[10]1
344	Not evaluated	SQ	51.7%	Rome III	SQ	Solhpour *et al*[55]
36/95 (ERD) 36/95 (NERD)	23/95	S, UE, OM, pH	17% (in ERD) 23% (in NERD) 39% (in FH)	Rome III	SQ	Lee *et al*[56]
207	Not evaluated	SQ	29.5%	Rome III	SQ	Kaji *et al*[36]1
286 (ERD) 74 (NERD)	Not evaluated	S, UE	11.2% 41.9%	Rome III	SQ	Noh *et al*[57]
2658	Not evaluated	S, UE	33.9%	Rome III Rome II	SQ	Yarandi *et al*[35]1
207	Not evaluated	SQ	29.5%	Rome III	SQ	Fujiwara *et al*[38]1
48/92 (NERD)	44/92	S, UE, OM22, M II-pH	39.6% (in NERD) 61.4% (in FH)	Rome III	SQ	Martinucci *et al*[39]1,2
1181 (ERD) 694 (NERD)	Not evaluated	S, UE	12.7% (in ERD) 18.3% (in NERD)	ReQuest	SQ	Mönnikes *et al*[58]
6810	Not evaluated	SQ	60%	ReQuest	SQ	Fass *et al*[59]2
257	Not evaluated	SQ	58%	ReQuest	SQ	Bardhan *et al*[60]

[1]Articles listed in both tables 1 and 2; [2]Abstract only (publication type). GERD: Gastroesophageal reflux disease; FH: Functional heartburn; IBS: Irritable bowel syndrome; S: Symptoms; SQ: Symptom questionnaire; PE: Physical examination; HE: Hematological examinations; BE: Barium enema; BC: Bacteriological culture; SC: Stool culture; BT: Lactose/lactulose breath test; AU: Abdominal ultrasonography; UE: Upper endoscopy; LE: Lower endoscopy; LEB: Lower endoscopy and biopsies; SBB: Small-bowel biopsies; Sg: Sigmoidoscopy; OM: Esophageal manometry; pH: pH-metry; MII-pH: pH impedance monitoring; ERD: Erosive reflux disease; NERD: Nonerosive reflux disease.

and IBS have proposed that visceral hypersensitivity, motility dysfunctions, and central neural mechanisms can be the main common pathophysiological mechanisms[11,13,61]. However, following the release of Rome Ⅲ criteria, an increasing number of studies have indicated the importance of a careful categorization of GERD patients *via* pathophysiological investigations to better appreciate the degrees of overlap between IBS and reflux symptoms in various subgroups of patients[39,56,62,63]. Accordingly, this section intends to appraise and critically discuss the available evidence supporting a pathophysiological relationship among GERD, FH and IBS. When attempting such a difficult task, two important points must be carefully considered: (1) In previous studies, GERD and IBS patients have been investigated to determine their pathophysiological and clinical features, while FH patients constitute a "new entity" for which pathophysiological studies are urgently required; and (2) Most of the available literature on the pathophysiology of FH addresses patients who were identified using old criteria (*i.e.*, criteria that have since been replaced by the Rome Ⅲ classification) that also identified NERD patients with normal esophageal AET. Even when these issues are kept in mind, IBS and FH, as well as IBS and GERD, appear to share some pathophysiological features that need to be carefully considered.

Visceral hypersensitivity

Most FDD patients display a reduced pain or discomfort threshold in response to visceral stimulation, implying that they might perceive a stimulus as uncomfortable or painful at significantly lower intensity than normal subjects would[64]. Such increased sensitivity can be usually documented throughout the whole gastrointestinal tract, suggesting diffuse, rather than site-dependent, involvement[65].

Studies aimed at gaining pathophysiological insights irrespective of the dominant digestive disorder have extensively investigated visceral hypersensitivity to a variety of stimuli (*e.g.*, acid perfusion, balloon distension, electrical stimulation) within both IBS[66] and GERD[63]. In particular, current data suggest that NERD patients displays equivalent or increased degrees of visceral hypersensitivity as compared with ERD, but may have lower levels than those shown by patients with functional esophageal disorders (*i.e.*, FH/chest pain of presumed esophageal origin). According to recent advances in basic science, three main mechanisms are believed to underlie visceral hypersensitivity (*i.e.*, peripheral sensitization, central sensitization and psychoneuroimmune interactions), and all of these have been documented in NERD patients[63]. Nevertheless, these factors' respective roles and degrees of involvement in the pathophysiology of FH remain to be established, particularly in the light of the Rome Ⅲ criteria. To verify whether FH patients have visceral hypersensitivity and to assess whether this feature is a common trait in IBS patients, some studies have investigated the presence of esophageal sensitivity to chemical or mechanical stimuli in FH and/or IBS patients.

Rodriguez-Stanley *et al*[67] reported that 89% of patients with FH (Rome Ⅱ) experienced abnormal responses to intraesophageal acid perfusion (Bernstein test), esophageal balloon distension, or both. In repeated studies using either esophageal balloon distension or electrical stimulation, patients with FH (Rome Ⅱ) have consistently demonstrated a lower perception threshold for pain or discomfort compared with patients with erosive esophagitis and/or abnormal 24-h esophageal pH monitoring[68,69]. Recently, Thoua *et al*[62] observed that patients with NERD had higher sensitivity to esophageal acid exposure than did ERD patients and controls, and this hypersensitivity was most pronounced with proximal esophageal acid exposure. Moreover, FH patients (Rome Ⅲ) were more hypersensitive to excess acid exposure than NERD patients were. Of note, these authors carefully selected patients with unequivocal reflux, taking care to exclude those with minor mucosal breaks, and the condition of hypersensitivity was found to be independent from motility changes[62]. Yang *et al*[70] found that cortical evoked potentials latencies induced by balloon distension were shorter in FH patients (Rome Ⅱ) than in controls before acid perfusion, and such perfusion decreased the latencies and increased their amplitude in FH patients, but not in controls. These findings suggest that dysfunctions of visceral neural pathways and/or alterations in cortical processing might generate and mediate esophageal hypersensitivity in FH.

Consistent with the notion that visceral hypersensitivity is not site-specific, Costantini *et al*[71] reported that during esophageal provocative testing (balloon distension and bethanechol administration), IBS patients displayed a lower threshold for esophageal symptoms compared with healthy volunteers, without any evident alteration of esophageal motility or decrease in esophageal basal pressure. In line with these observations, Trimble *et al*[72] demonstrated that IBS patients had a lower rectal sensory threshold for pain compared with healthy controls and that IBS patients displayed concomitantly lower sensory thresholds for both esophageal perception and discomfort evoked by balloon distension.

Whether the types of sensory dysfunctions previously detected in FH patients (Rome Ⅱ)[68] can also be observed in FH patients diagnosed in accordance with Rome Ⅲ criteria remains to be established. When investigating this issue, it must be considered that at present, there is not a unanimous consensus on how to define and measure the condition of lowered visceral threshold. A further critical issue is that visceral thresholds for different stimuli do not necessarily display parallel alterations. In this context, some relevant questions still await conclusive answers: (1) Which is the most meaningful index of an altered sensory threshold? (2) Can different stimuli be regarded as equivalent in nature? and (3) Considering day-to-day variations in the occurrence of symptoms, is there also a day-to-day variation in the underlying biological abnormalities responsible for these symptoms? Overall, great caution will be required in future studies addressing the pathophysiological meaning of visceral hypersensitivity in GERD/FH and/or IBS.

Motility dysfunction

Motor abnormalities might represent a common pathophysiological mechanism between GERD and IBS[61]. Consistent with this concept, some authors speculate that an overall dysfunction of smooth muscle throughout the GI tract might explain the overlap between IBS and GERD[22].

Of note, the pattern of esophageal motility has been shown to differ between ERD and NERD patients[73], while no significant differences have been found in LES pressure or contraction amplitude when comparing FH patients (Rome Ⅲ) to NERD patients with pathological AET[62]. In unclassified subjects complaining of heartburn, Bhalla *et al*[74] observed that acid infusion elicited an increase in symptom sensitivity in concomitance with a perturbation of esophageal contractility, as revealed by a greater increase in contraction amplitude, contraction duration, muscle thickness, and the incidence of sustained esophageal contractions during the second acid infusion in comparison with the first one.

To date, the possible contribution of motility dysfunction to the pathophysiology of FH remains unclear; however, while studying 12 unclassified subjects with heartburn using 24-h pH-metry, synchronized pressure

recording and high-frequency intraluminal ultrasound imaging of the oesophagus, Pehlivanov *et al*[75] highlighted a close correlation between heartburn episodes (whether associated with acid reflux or not) and abnormally long longitudinal muscle contraction durations. This motor correlate might also be relevant to a better understanding of the pathophysiological bases of heartburn perception in FH patients, but it has been documented only by a preliminary investigation and requires additional studies to be confirmed. Likewise, whether esophageal and bowel motor abnormalities occur concomitantly in patients with overlapping GERD/FH and IBS is currently unclear, and studies addressing this issue are required.

Central neural mechanisms

In FH patients, heartburn has been proposed to originate from factors other than luminal stimuli[68]. It has been speculated that central neural mechanisms related to psychological comorbidity (anxiety, depression and stress) could modulate esophageal perception and make patients prone to perceiving low-intensity esophageal stimuli as painful[69]. In particular, anxiety has been implicated as a factor that may modulate the degree of sensitization to esophageal acid testing[76].

Johnston *et al*[77] studied 101 patients with heartburn using esophageal pH monitoring. The subjects who showed no correlation between symptoms and refluxes displayed significantly higher levels of trait anxiety compared with patients with a positive correlation. Along the same line, Rubenstein *et al*[78] observed that in subjects with heartburn, esophageal sensation to both acid perfusion and mechanical distension was associated with increased levels of psychiatric distress and a diagnosis of IBS.

According to Posserud *et al*[79], no clear relationship between pain threshold and IBS symptoms (severe pain, bloating and diarrhea) has been convincingly established, and other mechanisms, including central nervous ones, are likely to play a relevant role. In line with this contention, Elsenbruch *et al*[80] observed that IBS patients can indeed experience a higher severity of distension-induced pain and overall discomfort despite unaltered rectal sensory thresholds, suggesting that the perception of visceral stimuli could be influenced by emotional factors. In contrast, it remains unclear what psychological factors are relevant for visceral hyperalgesia in IBS patients and how they may interact with biological mechanisms, such as peripheral/central neuroendocrine and immune processes[66].

Another aspect that deserves attention addresses the possible impact of sleep disorders on the pathophysiology of FDD symptoms. Jung *et al*[10] observed that self-reported insomnia and frequent abdominal pain represent two risk factors for IBS-GERD overlap compared with IBS or GERD alone. In addition, a positive association has been found between the severity of IBS symptoms and the severity of sleep disturbances. However, the pathophysiological mechanisms underlying this association are only partly understood. One possibility is that sleep disorders induce visceral hyperalgesia, thus amplifying the patient's perception of gastrointestinal symptoms[81,82].

Response patterns to drugs that modulate visceral pain

Pathophysiological similarities among GERD, FH and IBS might reflect similarities in their response patterns to the drugs that influence common pathophysiological mechanisms. According to the Rome Ⅲ criteria, FH patients' symptoms do not improve with PPI therapy. Consistent with this criterion, even before Rome Ⅲ, some authors reported that adding or switching PPIs to a visceral pain modulator [(*i.e.*, tricyclic antidepressants (TCAs) or selective serotonin reuptake inhibitors (SSRIs)] might induce beneficial effects in FH patients (Rome Ⅱ)[83]. Peghini *et al*[84] were the first to report that imipramine can reduce esophageal pain perception in healthy male volunteers. Clouse *et al*[85] investigated the effects of low-dose trazodone in patients with symptomatic esophageal dismotility and obtained a significantly greater global symptom improvement compared with placebo. Broekaert *et al*[86] observed that citalopram lowered chemical and mechanical esophageal sensitivity in healthy subjects without altering motility. Likewise, in a randomized placebo-controlled study, citalopram 20 mg/d was found to be effective in a selected group of patients with hypersensitive esophagus (*i.e.*, normal AET, positive SI)[87]. Overall, the current evidence, although preliminary in nature, suggests that SSRIs may exert beneficial effects in lowering esophageal sensitivity to chemical and mechanical stimuli. These observations encourage the performance of studies aimed at assessing the efficacy of SSRIs in patients with esophageal hypersensitivity. In this regard, it is interesting to note that antidepressants (*e.g.*, TCAs and SSRIs) have been found more effective than placebo in IBS treatment, as indicated by a recent review and meta-analysis of randomized controlled trials[88]. Thus, based on current knowledge, it can be tentatively speculated that visceral hypersensitivity might be a common trait among patients with esophageal hypersensitivity and/or IBS and that such an underlying pathophysiological condition might explain the beneficial responses to antidepressants in both these disorders. Overall, a critical appraisal of current evidence highlights the need for future clinical studies aimed at assessing the possible transverse beneficial actions of drugs in patients with concomitant ERD, NERD or FH and IBS. To date, it can be hypothesized that antidepressants have a beneficial role as visceral pain modulators.

CONCLUSION

In the present review, we have attempted to appraise and critically discuss whether the current literature supports an association between GERD and IBS and between FH and IBS. Our literature search highlights a high heterogeneity in terms of both the criteria and diagnostic procedures used to investigate the presence of heartburn

and IBS. In particular, most of the current epidemiological data do not rely on a formal diagnostic assessment of IBS and/or GERD; rather, the studies generally evaluated these disorders *via* symptom questionnaires. Another critical issue is the inclusion of patients with concomitant IBS and GERD without any attempt to distinguish FH from GERD using pathophysiological investigations. Indeed, a very few small studies have documented an actual concomitance of FH and IBS. The main reason for this paucity of data stems from the fact that, until the release of the Rome Ⅲ criteria, FH was not regarded as a distinct entity and was included in the same category as GERD. Moreover, most of current pathophysiological data refer to FH patients as defined by criteria older than the Rome Ⅲ classification. Accordingly, clear evidence of an association between IBS and FH, as defined by the Rome Ⅲ criteria, is presently lacking.

Independent of these critical issues, there is some evidence, though scarce and preliminary, of the concomitance of FH and IBS. In support of this contention, some studies have shown that FH and IBS may share common pathophysiological mechanisms, such as visceral hypersensitivity, and that drugs that act as visceral pain modulators (such as antidepressants) may exert beneficial effects on both disorders when tested in separate trials.

Overall, current knowledge about the GERD/FH and IBS overlap needs to be expanded *via* investigations based on updated diagnostic criteria, more accurate pathophysiological classifications, and careful categorization of patients with heartburn. To achieve these goals, future epidemiological and pathophysiological studies should be designed to properly assess the presence and extent of overlaps linking IBS with FH and various subgroups of GERD patients. In this context, it is also expected that a better pathophysiological characterization of heartburn will foster the identification of therapeutic strategies that target the common pathogenic mechanisms underlying FH and IBS.

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REVIEW

Criteria for the diagnosis and severity stratification of acute pancreatitis

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Abstract

Recent diagnostic and therapeutic progress for severe acute pancreatitis (SAP) remarkably decreased the case-mortality rate. To further decrease the mortality rate of SAP, it is important to precisely evaluate the severity at an early stage, and initiate appropriate treatment as early as possible. Research Committee of Intractable Diseases of the Pancreas in Japan developed simpler criteria combining routinely available data with clinical signs. Severity can be evaluated by laboratory examinations or by clinical signs, reducing the defect values of the severity factors. Moreover, the severity criteria considered laboratory/clinical severity scores and contrast-enhanced computed tomography (CE-CT) findings as independent risk factors. Thus, CE-CT scans are not necessarily required to evaluate the severity of acute pancreatitis. There was no fatal case in mild AP diagnosed by the CE-CT severity score, whereas case-mortality rate in those with SAP was 14.8%. Case-mortality of SAP that fulfilled both the laboratory/clinical and the CE-CT severity criteria was 30.8%. It is recommended, therefore, to perform CE-CT examination to clarify the prognosis in those patients who were diagnosed as SAP by laboratory/clinical severity criteria. Because the mortality rate of these patients with SAP is high, such patients should be transferred to advanced medical units.

Key words: Severe acute pancreatitis; Severity score; Scoring system; Prognostic factors; Case-mortality

Core tip: The new severity criteria of acute pancreatitis (AP) consist of two independent prognostic factors; laboratory and/or clinical severity scores and contrast-enhanced computed tomography (CE-CT) findings. Mortality rate of severe acute pancreatitis (SAP) that

satisfied both laboratory/clinical and CE-CT severity criteria was as high as 30.8%. It is recommended to perform CE-CT examination in those patients who were diagnosed as SAP by laboratory/clinical severity criteria. Patients who fulfill both severity criteria should be transferred to advanced medical units. The revised criteria are extremely useful to detect SAP at an early stage of AP.

Otsuki M, Takeda K, Matsuno S, Kihara Y, Koizumi M, Hirota M, Ito T, Kataoka K, Kitagawa M, Inui K, Takeyama Y. Criteria for the diagnosis and severity stratification of acute pancreatitis. *World J Gastroenterol* 2013; 19(35): 5798-5805 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5798.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5798

INTRODUCTION

Acute pancreatitis (AP) involves various clinical features from mild cases with only transient abdominal symptoms to severe fatal cases. It is important to identify patients with AP who are at risk for developing persistent organ failure early in the course of the disease[1]. Because case-mortality rate of severe AP (SAP) at the survey conducted by the Research Committee of Intractable Diseases of the Pancreas (RCIDP) supported by the Japanese Ministry of Health, Labour and Welfare was as high as 30%[2], SAP has been designated as an intractable disease by the Japanese Ministry of Health and Welfare since 1990, and the cost of treatment for SAP is paid in full by the government[3]. With the start of the medical expense payment system for patients with SAP, the RCIDP established the criteria for the diagnosis and severity stratification of AP. The severity scoring system was revised and the 2002 version was developed in 2002 (JPN criteria 2002)[1,4-6].

The criteria 2002 were complicated and composed of 18 items of prognostic factors; 5 clinical sign items, 10 blood test items, computed tomography (CT) findings, the presence of systemic inflammatory response syndrome (SIRS) and age[1,4-6]. The attending physician cannot remember all or even most of the factors. Moreover, these numerous parameters are not available soon enough or not available as the routine laboratory tests at all hospitals. There is a possibility, therefore, that incomplete examinations or defect values of the prognostic factors underestimated the severity of AP, resulting in insufficient and inadequate treatment of the disease, and aggravated AP[1]. There is another possibility that incomplete severity evaluation of AP overlooked the predicted serious cases to transfer to medical institutions with the high-level medical facilities and intensive care.

To decrease the mortality rate of the SAP, it is important to precisely evaluate the severity early in the disease and initiate appropriate treatment as early as possible[7-9]. The Ranson[10] and the modified Glasgow (Imrie) scores[11] represent a major advantage in the evaluation of the disease severity in AP but require 48 h of data collection before the severity can be evaluated. Thereafter, several clinical scoring systems such as acute physiology and chronic health evaluation (APACHE Ⅱ) score systems[12-15], SIRS[16], bedside index for severity in acute pancreatitis (BISAP)[17] and harmless acute pancreatitis score (HAPS)[18,19] for evaluating AP have been developed, but these methods to predict the development of SAP are complicated, cumbersome, and insufficiently sensitive[20]. Recently a web-based consultative process involving multiple international pancreatic societies revised and updated the Atlanta classification of AP[21-23]. Severity of the disease is classified as mild, moderate, and severe by the absence or presence of organ failure and local or systemic complications. Moderately SAP has transient organ failure of < 2 d, while SAP is defined by the presence of persistent organ failure for $\geqslant 2$ d. Although the revised Atlanta classification of AP is simple and will help the clinician to predict the outcome of patients with AP, it is unable to differentiate between moderately SAP and SAP before 48 h after onset. It is expected, therefore, to develop simpler severity scoring system with routinely available data that predicts outcome, the system that clinicians can use at the bedside.

PROBLEM OF THE PREVIOUS JPN CRITERIA FOR THE DIAGNOSIS AND STRATIFICATION OF THE SEVERITY

JPN clinical criteria for the diagnosis of AP proposed in 2002 are (1) acute abdominal pain and tenderness in the upper abdomen; (2) elevated pancreatic enzyme levels in serum, urine or ascitic fluid; and (3) ultrasonographic (US) or radiologic abnormalities characteristic of AP[1,4-6]. When at least two of the above conditions are present, then excluding other pancreatic and acute abdominal diseases of different causes can make the diagnosis of AP. Acute exacerbation of chronic pancreatitis is also included in this category. When diagnosis is confirmed by surgery and/or autopsy, the event has to be duly recorded[1,4-6].

The JPN severity criteria 2002 consisted of 5 clinical sign items (shock, respiratory failure, mental disturbance, severe infection, hemorrhagic diathesis), 10 blood test items [base excess (BE), hematocrit (Ht), blood urea nitrogen (BUN) or creatinine, calcium concentration (Ca), fasting blood glucose, arterial oxygen saturation (PaO_2), lactate dehydrogenase (LDH), total protein, prothrombin time (PT), and platelet count], and CT findings. In cases with severity scores $\geqslant 2$ points, SIRS and an age over 70 had to be added to the prognostic factors[1,4-6]. These items of prognostic factors were all scored as severity scores, 1 or 2 points for each positive factor, and the highest possible total score was 27 points. However, blood glucose level, serum total protein concentrations and Ht are inappropriate for the prognostic factors after the initiation of the treatment of the disease because initial fluid resuscitation might have an influence on the

measurement value of these laboratory data. In addition, the severity criteria had redundant prognostic factors indicating similar clinical condition such as shock and the decrease in BE, and dyspnea and fall of PaO_2. Bleeding tendency, platelet counts and PT also indicate similar clinical condition. Moreover, the clinical signs such as severe infection that rarely develops within 48 h after disease onset were implicated in the severity criteria. The severity criteria included CT grade by the non-enhanced plain CT scan as one of the prognostic factors. Plain CT scans can evaluate peripancreatic inflammatory changes, but are unable to identify pancreatic necrosis that is closely associated with various complications and prognosis[21,24-26].

Usefulness of the JPN criteria 2002 for severity stratification was evaluated in 1131 consecutive patients with AP that had been admitted to high specific or intensive therapy units of the affiliated research group hospitals from January 1 1995 to December 31 1998 (survey 1998; before the establishment of the JPN criteria 2002), and in 1768 patients who visited the hospitals in the year 2003 (survey 2003; from January 1 to December 31; after the establishment of the criteria in 2002)[1,4-6]. The results revealed that the severity score have almost the same value for assessment as the APACHE Ⅱ score and the Ranson score[4].

In survey 1998, case-fatality rate of mild, moderate and SAP was 0.2%, 1.6% and 13.8%, respectively, whereas it was 0.1%, 0.7% and 9.0%, respectively, in survey 2003[1,4-6]. The case-mortality rate of mild and moderate AP was quite low, and there was little clinical significance to differentiate moderate from mild AP. The case-mortality rate of SAP at stage 2 (3.7%) was low compared with that at stage 3 (25.4%) in survey 2003[1,4-6]. Therefore, it was inappropriate to classify these patients at stage 2 as SAP and identify as applicants for the medical expense payment system[5].

Although the previous severity criteria classified prognostic factors into 2 groups; each of the items in the first group has 2 points, while that in the second group is 1[1,4-6], there is no significant difference in case mortality between these 2 groups with different prognostic scores. JPN severity criteria 2002 were complicated and included several prognostic factors which cannot be measured at outpatient clinic or emergency room, especially at night. In addition, multiple scoring systems of the severity criteria were very cumbersome to use and they suffer from their complexity[1,4-6]. Indeed, 56% of 1768 clinical records of AP in survey 2003 had defect values of more than 3 items of 11 laboratory examinations. Especially, BE was measured in only 25.1%, and PT and PaO_2 were measured in only 38.3% and 38.7%, respectively[1]. These results indicate that even if we can diagnose the patient as AP, in the presence of many defect values a correct stage classification is difficult, and it is very likely that we underestimate the severity.

Because CT grade was included as one of the prognostic factors[1,4-6], it was required to perform CT examination repeatedly to precisely evaluate the severity and stage of AP. However, it is unacceptable to perform CT examination repeatedly[27], and thus one of the prognostic factors remains as a defect value. In addition, there are many hospitals that cannot perform CT examination and laboratory tests such as PT, especially at night. This might be one of the reasons for many defect values in the clinical records of AP[1].

NEW DIAGNOSTIC CRITERIA OF AP

JPN diagnostic criteria of AP are revised taking into account of the recent progress of imaging studies and laboratory examinations of pancreatic enzymes. The revised clinical criteria for the diagnosis of AP are (1) acute pain and tenderness in the upper abdomen; (2) elevated pancreatic enzyme levels in in blood and/or urine; and (3) ultrasound (US), CT or magnetic resonance imaging (MRI) abnormalities of the pancreas characteristic of AP[1]. When at least two of the above conditions are present, the diagnosis of AP can be made by excluding other pancreatic and acute abdominal diseases of other causes than pancreatitis. Acute exacerbation of chronic pancreatitis is included in this category.

Measurement of pancreatic enzyme levels in serum has been generally adopted in clinical practice, whereas those in ascitic fluid and urine are rarely determined. Since, however, recent studies have demonstrated that urinary strip tests for trypsinogen activation peptide (TAP) and trypsinogen-2 provide a reliable early diagnosis of AP[28-35], the revised diagnostic criteria included the elevation of the pancreatic enzymes in serum and/or urine, excluding that in ascitic fluid. It is well known, however, that some patients with AP, mostly alcoholic etiology, show normoamylasemia[28], and that serum amylase level rises only slightly in many patients with acute exacerbations of chronic alcoholic pancreatitis[36]. Moreover, serum amylase level seldom rises in AP caused by hyperlipidemia[37,38] and in those with pancreatic insufficiency[39]. In addition, the elevation of serum amylase level is only transient and declines within 3 d after onset of AP[28,40]. On the other hand, abnormally high values of serum lipase persist for longer period than that of serum amylase and are observed even in cases of alcohol-induced pancreatitis[41]. Although a recent case report of AP has demonstrated that serum amylase and lipase remain normal throughout the acute phase of AP in a man with pancreatic insufficiency and cystic fibrosis[39], serum lipase is considered to be a more reliable diagnostic marker of AP than serum amylase. Therefore, the revised diagnostic criteria recommend determining pancreatitis specific enzymes in serum and/or urine such as pancreatic-type amylase[42,43] and lipase[44].

The new diagnostic criteria require the presence of clear findings indicating AP by imaging studies such as US, CT and MRI. US can visualize pancreatic enlargement, inflammatory changes around the pancreas, and abnormal findings associated with AP such as the presence of ascitic fluid and gallstones. US examination can

Table 1 Laboratory/clinical criteria for grading the severity of acute pancreatitis

No.	Laboratory/clinical criteria
1	Base excess ≤ -3 mEq/L or shock (systolic blood pressure ≤ 80 mmHg)
2	PaO_2 ≤ 60 mmHg (room air) or respiratory failure (artificial respiratory ventilation)
3	BUN ≥ 40 mg/dL or creatininee ≥ 2.0 mg/dL or oliguria (urinary volume ≤ 400 mL/d after hydration)
4	LDH: More than twice higher than the upper limit of normal (≥ 700 IU/L)
5	Serum total Ca ≤ 7.5 mg/dL
6	Platelet count ≤ $1 \times 10^5/mm^3$
7	CRP ≥ 15 mg/dL
8	Positive score of SIRS criteria ≥ 3
9	Age ≥ 70 yr

One point for each positive factor. Severe acute pancreatitis: total scores ≥ 3 points. BUN: Blood urea nitrogen; LDH: Lactate dehydrogenase; SIRS: Systemic inflammatory response syndrome; CRP: C-reactive protein.

Table 2 Contrast-enhanced computed tomography criteria for grading the severity of acute pancreatitis

Contrast-enhanced computed tomography criteria	Scores
Extension of extrapancreatic inflammatory changes	
Anterior pararenal extraperitoneal space	0 point
Root of the mesocolon	1 point
Beyond inferior renal pole	2 points
Unenhanced area in the pancreatic parenchyma	
(Divide the pancreas into 3 areas for expediency, head, body and tail)	
Limited to one area or peripancreatic area	0 point
Extend over 2 areas	1 point
More than 2 areas	2 points

Severe acute pancreatitis: total computed tomography severity scores ≥ 2 points.

be performed repeatedly at bedside. CT provides clear local images without being affected by the adipose tissue in the abdominal wall and abdominal cavity[14,45]. CT findings of an enlarged pancreas, inflammatory changes around the pancreas and fluid collections are useful marker for the diagnosis of AP. Thus, CT is the most important imaging procedures for the diagnosis of AP[46-48]. MRI scanning can also visualize the enlargement of the pancreas and the inflammatory changes around the pancreas[49,50].

SEVERITY CRITERIA OF AP BY MULTIPLE-SCORING SYSTEM

Following the correct diagnosis of AP, severity stratification should be performed promptly and repeatedly, in particular for the first 48 h after the onset of the disease[1]. Early recognition of severe disease and application of appropriate therapy require vigilance as decisions regarding management need to be made shortly after admission.

The revised severity score put the redundant factors that show similar clinical conditions together into one, and deleted the unclear clinical signs. Since the new severity criteria combined laboratory data with clinical signs, the severity of AP can be evaluated by one of these findings. BE can be substituted by shock (systolic blood pressure less than 80 mmHg), PaO_2 by respiratory failure (artificial respiratory ventilation), and BUN or creatinine by oliguria (urinary volume less than 400 mL/d after hydration). Thus, SAP can be properly diagnosed by reducing underestimation of severity by the defect values (Table 1).

Among several serum biochemical markers that have been developed for severity stratification of AP, C-reactive protein (CRP) remains the most useful[19,32,51-54]. Although its increase delays, peaking not earlier than 72 h after the onset of symptoms, it is accurate and widely available. According to United Kingdom guidelines for the management of AP[55] and the Working Party of the Program Committee of the Bangkok World Congress of Gastroenterology 2002[56], CRP ≥ 15 mg/dL is adopted as a prognostic factor. Moreover, Gardner *et al*[57] have demonstrated that an age above 70 years is an independent risk factor for mortality in patients admitted with SAP. Based on these previous studies, the new severity criteria included CRP and age of the patient. In spite of these changes, the new severity criteria that employ routinely available data are simple and easy to remember.

Since the contrast-enhanced CT (CE-CT) is the mainstay of imaging patients with AP and recommended for the evaluation of the severity of AP[20,24-27,34,35,49], the revised severity criteria included the CE-CT findings of the presence and extent of pancreatic necrosis, and the extent of peripancreatic inflammatory changes (Table 2). The revised Atlanta classification provided precise definitions of CE-CT findings, including peripancreatic necrosis, walled-off-necrosis and pseudocyst[21,22]. Although the revised Atlanta classification suggested that pancreatic necrosis can rarely be identified accurately during the first several days of hospitalization, CE-CT findings help us to decide special measures such as continuous regional arterial infusion (CRAI) of protease inhibitors and antibiotics, and continuous hemodiafiltration (CHDF)[58-60]. Once it is thought that contrast medium exacerbates pancreatitis[61-63], but denied by another studies[64,65]. Since, however, there is a possibility that intravenous contrast media extend pancreatic necrosis and exacerbate renal impairment[61-63], vigorous intravenous hydration for the purpose of intravascular resuscitation is important during and after CE-CT examination. Attending physicians must aware of the possibility that the contrast medium aggravates renal dysfunction associated with SAP.

The new severity criteria considered laboratory/clinical symptoms and radiographic features of CE-CT scans as independent risk factors. Indeed, Leung *et al*[14] have demonstrated that CT severity index is a useful tool in assessing the severity and outcome of AP, and superior to Ranson score[10] and APACHE II scoring system[12-15] in predicting AP outcome. Thus, the CE-CT is not necessarily required to evaluate the severity of the patients with AP. Preliminary study revealed that the case-fatality

Table 3 Verification of the revised severity criteria

Total severity score (points)	Revised severity criteria	Criteria 2002
0	66	77
1	51	31
2	18	15
3	11 (1)	9
4	4	7
5	4 (2)	6
6	2 (1)	3 (1)
7	0	2 (1)
8	0	0
9	0	1
10	0	2 (2)
11	0	2
12	0	1
Total	156 (4)	156 (4)

Results shown are number of patients. Number in parenthesis indicates patients died of acute pancreatitis. The same patients with acute pancreatitis were evaluated by the revised criteria and by the criteria 2002. Total severity score of the revised criteria ⩾ 3 points, while that of the criteria 2002 ⩾ 2 points was diagnosed as severe acute pancreatitis.

Table 5 Relationship between the laboratory/clinical and the contrast-enhanced computed tomography severity scores

Total CE-CT severity score (points)	Total laboratory/clinical severity score (points)							Total
	0	1	2	3	4	5	6	
0	0	0	0	0	0	0	0	0
1	56	40	13	5	1	0	0	115
2	3	5	2	1	0	2 (1)	1 (1)	14 (2)
3	2	1	1	3 (1)	3	2 (1)	1	13 (2)
Total	61	46	16	9 (1)	4	4 (2)	2 (1)	142 (4)

Results shown are number of patients. Number of patients died of acute pancreatitis is indicated in parenthesis. In these 142 patients, laboratory/clinical and contrast-enhanced computed tomography (CE-CT) examinations were evaluated at the same time.

in patients with the CE-CT severity score 1 was 3.3%, while that in those with severity score 2 and 3 points was 21.9% and 33.3%, respectively. Thus, the severity scores of CE-CT ⩾ 2 points was defined as SAP (Table 2).

Analysis of case records of 1337 consecutive patients with AP in survey 2003[1,5,6] in that more than 5 items of 9 prognostic factors of the new severity criteria were recorded revealed that case-fatality rate of patients with severity score point 0 and point 1 was nearly the same (0.2% *vs* 0.7%), whereas that of patients with severity score 2 and 3 points was greatly different (2.6% *vs* 11.1%). Thus, the new criteria divided the severity of AP into mild (severity score ⩽ 2 points) and SAP (severity score ⩾ 3 points). Based on this classification, case-mortality rate of mild AP and SAP was 0.83% (9/1183) and 19.5% (30/154), respectively.

VERIFICATION OF THE NEW SEVERITY CRITERIA

Usefulness of the new severity criteria was prospectively

Table 4 Relationship between the revised laboratory/clinical or contrast-enhanced computed tomography severity score and incidence of organ failure

Total severity score (points)	Incidence of organ failure	
	Laboratory/clinical	CE-CT
0	1.5%	0.0%
1	7.8%	4.3%
2	5.5%	42.9%
3	36.4%	46.2%
4	50.0%	-
5	75.0%	-
6	100.0%	-

Total number of patients evaluated by laboratory/clinical examinations was 156, whereas contrast-enhanced computed tomography (CE-CT) severity score was evaluated in 142 of these 156 patients at the same time.

studied in 156 patients with AP. CE-CT severity score was evaluated in 142 of these 156 patients at the same time with laboratory examinations. Overall case-mortality of 156 patients with AP was 2.6%, and was similar to that reported in nationwide survey in 2003[1,5,6]. Although some survey sheets had defect data of laboratory examinations, most frequently BE (defect value 41.0%) and PaO_2 (defect value 41.0%), these data were substituted by clinical signs of shock (defect value 0%) and respiratory failure (defect value 0%), respectively. Therefore, the severity score could be precisely calculated even if these laboratory data were defect values.

The revised severity criteria (Table 1) identified 13.5% of these 156 patients with AP as SAP, whereas 30.8% were diagnosed as SAP if the criteria 2002 were adopted. Case-mortality of SAP diagnosed by the revised criteria was 19.1%, whereas that by the criteria 2002 was only 8.3% due to large number of patients who are diagnosed as SAP (Table 3). The validity of the revised classification was further revealed by the incidence of complications of organ failure. Complications of organ failure were far greater in patients with SAP than in those with mild AP (Table 4). These results clearly indicate that the patients with SAP diagnosed by the revised criteria are suitable as applicants for the medical expense payment system[3].

Since the new severity criteria consider laboratory data/clinical symptoms, and the CE-CT severity score as independent risk factors, SAP can be diagnosed either by the laboratory/clinical severity criteria or by the CE-CT severity criteria. There was no fatal case of mild AP diagnosed by the laboratory/clinical severity score regardless of CT severity score. Similarly, there was no fatal case of mild AP diagnosed by the CE-CT severity score regardless of laboratory/clinical severity scores (Table 5). Case-mortality rate of patients with SAP diagnosed by the laboratory/clinical severity score was 21.1%, whereas that in those diagnosed by the CE-CT severity score was 14.8%. Case fatality of SAP that fulfilled both laboratory/clinical (severity score ⩾ 3 points) and CE-CT severity criteria (severity score ⩾ 2 points) was as high as 30.8%. It is recommended, therefore, to perform CE-CT examination to clarify the prognosis in patients who were diagnosed as SAP by laboratory/clinical severity score. Because the

mortality rate of these patients with SAP was high, such patients should be transferred to advanced medical units with physicians specializing in intensive care, endoscopic treatment, radiological intervention, and biliary-pancreatic surgery[1,5,6].

CONCLUSION

The new severity criteria consist of laboratory examinations combined with clinical symptoms and the CE-CT severity score. The laboratory and/or clinical symptoms and the CE-CT findings are independent risk factors. SAP can be diagnosed either by the severity score alone, or by the CE-CT findings alone. Mortality rate of SAP that fulfilled both laboratory/clinical and CE-CT severity criteria was high. It is recommended, therefore, to perform CE-CT examination in those patients who were diagnosed as SAP by laboratory/clinical severity criteria. Patients with SAP who fulfill both severity criteria should be transferred to advanced medical units. The revised criteria are extremely useful to detect SAP at an early stage of AP.

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MINIREVIEWS

Achalasia: A review of clinical diagnosis, epidemiology, treatment and outcomes

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Abstract

Achalasia is a neurodegenerative motility disorder of the oesophagus resulting in deranged oesophageal peristalsis and loss of lower oesophageal sphincter function. Historically, annual achalasia incidence rates were believed to be low, approximately 0.5-1.2 per 100000. More recent reports suggest that annual incidence rates have risen to 1.6 per 100000 in some populations. The aetiology of achalasia is still unclear but is likely to be multi-factorial. Suggested causes include environmental or viral exposures resulting in inflammation of the oesophageal myenteric plexus, which elicits an autoimmune response. Risk of achalasia may be elevated in a sub-group of genetically susceptible people. Improvement in the diagnosis of achalasia, through the introduction of high resolution manometry with pressure topography plotting, has resulted in the development of a novel classification system for achalasia. This classification system can evaluate patient prognosis and predict responsiveness to treatment. There is currently much debate over whether pneumatic dilatation is a superior method compared to the Heller's myotomy procedure in the treatment of achalasia. A recent comparative study found equal efficacy, suggesting that patient preference and local expertise should guide the choice. Although achalasia is a relatively rare condition, it carries a risk of complications, including aspiration pneumonia and oesophageal cancer. The risk of both squamous cell carcinoma and adenocarcinoma of the oesophagus is believed to be significantly increased in patients with achalasia, however the absolute excess risk is small. Therefore, it is currently unknown whether a surveillance programme in achalasia patients would be effective or cost-effective.

Key words: Epidemiology; Achalasia; Incidence; Treatment; Oesophageal cancer risk

Core tip: Achalasia remains a disease of unknown aetiology. Multicentre studies could help elucidate the cause, especially as it presents with a similar phenotype to Chagas disease which is much better understood. Improved understanding of aetiology could guide novel treatments. Current treat choice in fit patients lies between pneumatic dilatation and laparoscopic Heller's myotomy. Botulinum toxin is appropriate and effective for those unfit for other intervention. Novel treatments such as metal stents and natural orifice surgery are being trialled.

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INTRODUCTION

Achalasia is a motility disorder of the oesophagus, of unknown aetiology, which results in degeneration of the

myenteric nerve plexus of the oesophageal wall. The resultant abnormalities and diagnostic characteristics of achalasia include loss of oesophageal peristalsis and failure of relaxation of the lower oesophageal sphincter, particularly during swallowing[1].

DIAGNOSIS

Dysphagia is the cardinal symptom of achalasia. Diagnosis requires a high index of suspicion and exclusion of other causes. Diagnosis is confirmed by manometric, endoscopic and radiographic investigations. Oesophageal manometry is regarded as the gold standard in the diagnosis of achalasia, classically showing aperistalsis and failure of relaxation of the lower oesophageal sphincter[2], as shown in Figure 1. Endoscopy is not accurate in the diagnosis of achalasia. However, it is still necessary to exclude a carcinoma at the lower end of the oesophagus[3]. Barium esophagogram can often show the pathognomonic "bird's beak" appearance of the distal oesophagus with dilatation of the oesophagus proximally (Figure 2). However, this is often a finding in established disease and therefore a normal barium swallow does not rule out the diagnosis of achalasia. With the introduction of high resolution manometry, together with pressure topography, plotting the diagnosis of achalasia can now be classified into three subtypes; type 1 classic achalasia, type 2 achalasia with compression and pressurisation effects, and type 3 spastic achalasia[4]. This classification process can aid treatment decisions, with type 2 achalasia being the most responsive to pneumatic dilatation, Hellers myotomy and botulinum toxin and therefore having the best outcome[5]. Oesophageal emptying is determined by the distensibility of the oesophago-gastric junction. This can be assessed using an endoscopic functional luminal imaging probe (EndoFLIP). Recently, Dutch and American groups have demonstrated that this novel technique is a better predictor than lower oesophageal sphincter pressure for assessing response to treatment in achalasia, both symptomatically and when measured by gastric emptying by oesophageal emptying[6,7].

PATHOGENESIS

The pathogenesis of achalasia is not well understood but it is believed to be due to an inflammatory neurodegenerative insult with possible viral involvement. The measles and herpes viruses have been suggested as candidate viruses however molecular techniques have failed to confirm these claims and therefore the causative agent remains undiscovered[8]. Genetic and autoimmune components have also been suggested as origins of the neuronal damage however research to date has not found the exact cause[9]. Inflammatory changes within the oesophagus following the causative insult result in the loss of postganglionic inhibitory neurons in the myenteric plexus and a consequent reduction in the inhibitory transmitters, nitric oxide and vasoactive intestinal peptide. The excitatory

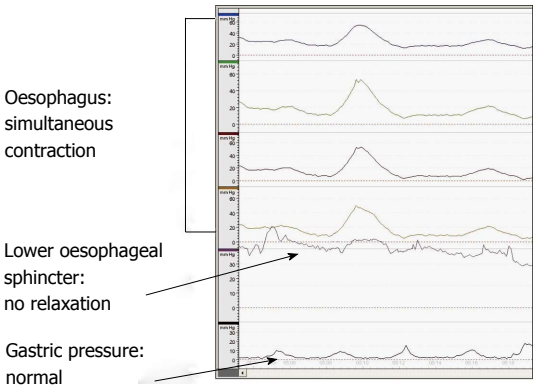

Figure 1 Oesophageal manometry demonstrating simultaneous contractions within the oesophagus and a non-relaxing lower oesophageal sphincter.

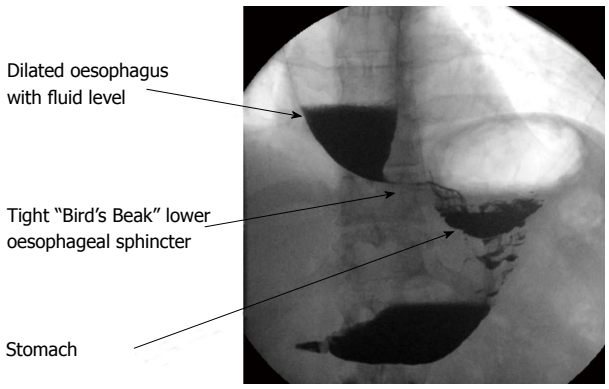

Figure 2 Barium swallow demonstrating typical "bird's-beak" appearance of the lower oesophageal sphincter in achalasia. The oesophagus above this is dilated.

neurons remain unaffected, with the resulting imbalance between excitatory and inhibitory neurons preventing lower oesophageal sphincter relaxation[10]. Lack of peristalsis and a non-relaxing lower oesophageal sphincter cause progressive dysphagia. Regurgitation, particularly at night, with aspiration of undigested food and weight loss can be presenting features, particularly in established disease. Features which present in the early stages of the disease may be similar to that of gastro-oesophageal reflux, including retrosternal chest pain typically after eating and heartburn[11]. Due to initial non-specific symptoms in early stage disease and the low prevalence of achalasia worldwide, the condition often goes undiagnosed for many years, giving rise to features of late stage disease and their associated complications.

EPIDEMIOLOGY

Achalasia is a relatively rare condition. A summary of studies published to date on achalasia incidence and prevalence is shown in Table 1[12-25]. The incidence of achalasia varied between studies, with reports as low as 0.03/100000 per year in Zimbabwe[22] to 1.63/100000 per year in Canada[14]. The majority of incidence rates re-

Table 1 Summary of epidemiological studies of achalasia incidence and prevalence in adults

Study	Location	Years studied	Total number of achalasia patients	Prevalence rate (per 100000)	Incidence rate (per 100000/year)
Howard *et al*[12]	Edinburgh, Scotland	1986-1991	Not reported	Not reported	0.81
Birgisson *et al*[13]	Iceland	1952-2002	62	8.7	0.55
Sadowski *et al*[14]	Alberta, Canada	1995-2008	463	2.51[8]	Not reported
				10.82[9]	1.63[9]
Mayberry *et al*[15]	Great Britain and Ireland	1972-1983	6306	Not reported	Not reported
	Scotland		583	11.2	1.1-1.2[6]
	Wales		197	7.1	Not reported
	Northern Ireland		153	9.8	Not reported
	Republic of Ireland		453	13.4	Not reported
	England		4920	10.8	0.9[7]
Mayberry *et al*[16]	Cardiff, Wales	1926-1977	48	Not reported	0.4
Mayberry *et al*[17]	Nottingham, England	1966-1983	53	8.0	0.51
Arber *et al*[18]	Israel	1973-1983	162	7.9[1]	0.8[3]
				12.6[2]	1.15[4]
Earlam *et al*[19]	Rochester, United States	1925-1964	11	Not reported	0.6
Galen *et al*[20]	Virginia, United States	1975-1980	31	Not reported	0.6
Mayberry *et al*[21]	New Zealand	1980-1984	152	Not reported	1.0
Stein *et al*[22]	Zimbabwe	1974-1983	25	Not reported	0.03
Farrukh *et al*[23]	Leicester, England	1986-2005	14	Not reported	0.89[5]
Ho *et al*[24]	Singapore	1989-1996	49	1.77	0.29
Gennaro *et al*[25]	Veneto, Italy	2001-2005	365	Not reported	1.59

[1]Rate in 1973 only; [2]Rate in 1983 only; [3]Rate between 1973-1978; [4]Rate between 1979-1983 only; [5]Rate only applicable for South Asian population of region; [6]Rate reported as 1.1 for men and 1.2 for women; [7]Rate only applicable to Oxford region of England; [8]Rate in 1996 only; [9]Rate in 2007 only.

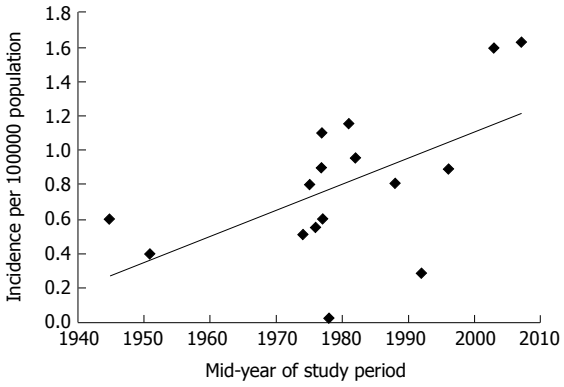

Figure 3 Achalasia incidence by mid-study time points.

ported clustered between 0.5-1.2 per 100000/year (Table 1). In an attempt to investigate changing incidence rates over time, we plotted incidence rates against the mid-timepoint within the study periods (Figure 3). As shown in Figure 3, incidence rates of achalasia appear to be rising, with most reports since the 1980s exceeding rates of 0.8/100000 per year, which has doubled to 1.6/100000 per year in post-2000 studies. Whether this reflects a true rise in incidence, or greater awareness and improved diagnosis of the condition remains uncertain though.

There are no distinct patterns of achalasia incidence in terms of age and sex distribution; it can affect both genders, all races and all ages[26]. A few studies have suggested a bimodal distribution of incidence by age, with peaks at around age 30 and 60 years[12,18,24], while others have pointed towards a generally increased risk of achalasia with increased age[15,23,25]. Achalasia appears to affect males and females to largely equal extents[12,13,15,18,21,23-25,27] although some investigations have detected slightly higher rates amongst females[16,19,28]. Only one study reported a higher achalasia incidence in men[14]. A study carried out by Mayberry *et al*[15] found achalasia to be significantly more common in the Republic of Ireland in comparison to its neighbouring countries (Table 1). Similarly, a study which reviewed the incidence of achalasia in New Zealand found differing incidence between ethnic groups[21]. The Pacific Islanders had an incidence of 1.3/100000 per year in comparison to those of Maori descent having an incidence of 0.2/100000 per year. This may reflect the influence of genetic factors in achalasia aetiology[21].

A Canadian population-based study also considered the prevalence and survival rates of patients with achalasia[14]. Sadowski *et al*[14] found that the prevalence of achalasia rose from 2.51/100000 in 1996 to 10.82/100000 in 2007, despite a relatively stable incidence over the same time period (Table 1). The rise in prevalence was seen in both genders but was noted to be more pronounced in males, reflecting the fact that achalasia is a slowly progressive disease. This rise in prevalence was also evident in an Israeli study[18] and was noted in an Icelandic study between 1952 and 2002[13]. It is interesting to note that the Canadian study observed survival of achalasia patients to be significantly lower than the age-sex matched control population[14]. However, others have discerned that the majority of deaths in achalasia patients result from unrelated causes, leading to an equivalent life expectancy to the general population[29].

AETIOLOGY

There has been much debate over the aetiology of acha-

lasia, with several potential triggers for the inflammatory destruction of inhibitory neurons in the oesophageal myenteric plexus being implicated. These include autoimmune responses, infectious agents and genetic factors.

Auto-immune conditions

One recent study observed that patients with achalasia were 3.6 times more likely to suffer an autoimmune condition, compared with the general population[30]. Sjogren's syndrome, Systemic Lupus Erythematosus and uveitis were all significantly more prevalent in achalasia patients. The study also found the presence of a T-cell infiltrate and antibodies within the myenteric plexus of many patients with achalasia and an increased presence of human leukocyte antigen class II antigens[30]. Another study noted an overall higher prevalence of neural autoantibodies in patients with achalasia in comparison with a healthy control group[31]. Although no specific autoantibody was identified, this further supports the theory that achalasia has an autoimmune basis[31].

Infectious agents

The role of an infectious agent in the development of achalasia has been widely debated with several viral agents being implicated. For example, Chagas disease has a known infectious aetiology, and exhibits many similarities with achalasia[32]. In addition, there are several reports of varicella zoster virus and Guillain-Barre syndrome preceding the onset of achalasia[32]. Antibody studies have demonstrated increased titres to herpes and measles viruses in patients with achalasia in comparison to healthy control groups[33,34]. One study looking specifically at the link between the herpes simplex virus (HSV) and primary achalasia indicated the presence of HSV-1 reactive immune cells in the lower oeseophageal sphincter of achalasia patients, suggesting that HSV-1 may be involved in the neuronal damage to the myenteric plexus leading to achalasia[35]. A further study of peripheral blood immune cells found that patients with achalasia showed an enhanced response to HSV-1 antigens[34]. In contrast, another investigation using PCR on myotomy specimens did not find any association between herpes, measles or human papilloma viruses and achalasia[36]. The current evidence for a causative infectious agent is contradictory and no clear causal relationship has yet been established.

Genetic predisposition

The genetic basis for achalasia has not been widely investigated due to its low prevalence. One syndrome, known as the triple "A" syndrome, which consists of a triad of achalasia, alacrima and adrenocorticotrophic hormone resistant adrenal insufficiency is a known autosomal recessive disorder caused by gene mutations on chromosome 12. This syndrome, together with the prevalence of cases within children of consanguineous couples[37], suggests the possibility for a genetic component to the aetiology of achalasia. There have been associations with other genetic diseases including Parkinson's disease, Downs syndrome and MEN2B syndrome[10]. One recent suggested the possibility of involvement of the rearranged during transfection gene, which is a major susceptibility gene for Hirschprung's disease (also linked with Down's syndrome)[9]. Mayberry *et al*[38] conducted a study of first degree relatives of achalasia patients but concluded that inheritance was unlikely to be a significant causative factor due to the rarity of familial cases and exposure to common environmental and social factors within a family group may explain the presence of familial cases of achalasia.

It has been postulated that achalasia may incorporate a multi-factorial aetiology with an initiating event such as a viral or environmental insult resulting in oesophageal myenteric plexus inflammation. This inflammatory reaction may then initiate an autoimmune response in a susceptible group of genetically predisposed people, causing destruction of inhibitory neurons[39].

TREATMENT

The mainstay of treatment for achalasia is either pneumatic balloon dilatation or laparoscopic myotomy[40]. In pneumatic balloon dilatation, a balloon is positioned across the lower oesophageal sphincter and inflated, effectively rupturing the muscle of the affected segment. Surgical myotomy can be performed as either an open or laparoscopic procedure. The laparoscopic technique is now the most commonly performed. The procedure involves making a longitudinal division of the circular muscle of the lower oesophageal sphincter, extending this both proximally and distally onto the cardia[11]. Many surgeons advise the use of an anti-reflux procedure together with surgical myotomy, as these patients at are an increased risk of reflux following surgery[3]. A recent study compared partial anterior and partial posterior fundoplication following cardiomyotomy. It concluded that partial posterior fundoplication was superior to the anterior procedure due to significantly lower reintervention rates for postoperative dysphagia[41].

The best comparative study between pneumatic dilatation and surgery to date has demonstrated remarkably similar outcomes in matched patients over a three year follow up period[42]. Therapeutic success at two years was noted in 86% of those treated by pneumatic dilatation and 90% of those who had laparoscopic Heller's myotomy. The regimen for pneumatic dilatation was rigorous with the option of multiple dilatations. The accompanying editorial suggests that choice should be determined by patient preference and local expertise[43]. A new endoscopic esophagomyotomy technique has been recently introduced: Peroral endoscopic myotomy involves dividing the inner circular muscle of the oesophagus. This requires sophisticated expertise and remains experimental[39].

In patients for whom invasive procedures are not suitable, alternative treatment options may be considered including pharmacological intervention using long-acting

nitrates and calcium channel blockers. However, these are of limited benefit[44]. A further option is botulinum toxin injection into the lower oesophageal sphincter. This technique offers good short term results[45]. Most recently, metal stents have been used successfully[46]. Both of these options are generally only suited to those with several co-morbidities[9].

COMPLICATIONS

Patients with achalasia are at risk of developing the complications associated with disease progression as well as its treatment interventions. The complications of achalasia that can develop as a result of the natural course of the condition include aspiration-pneumonia[47]. Megaesophagus develops in 10% of inadequately treated cases and can ultimately require oesophagectomy[48].

Squamous cell carcinoma is the most common oesophageal cancer in patients with achalasia and this is thought to result from the high level of nitrosamines produced by bacterial overgrowth due to stasis in the oesophagus leading to chronic inflammation and dysplasia[49]. There is considerable variation in the documented oesophageal cancer risk in achalasia. One review found that the prevalence of oesophageal cancer in achalasia was 3%, corresponding to a 50-fold increased risk[50], while a prior review reported increased risks ranging from 0-33-fold greater than the general population[51]. Subsequent reports would also indicate a slightly more modest, but still significantly elevated, risk of oesophageal cancer 16-28-times greater than an age-sex matched control population[52,53].

The risk of oesophageal adenocarcinoma is also increased in those with achalasia and may be a complication of longstanding reflux following successful interventional treatment[27,54]. A recent publication from The Netherlands demonstrated that 8.2% of 331 primary achalasia patients developed Barrett's oesophagus over a period of up to 25 years[55]. Two of these Barrett's cases progressed to develop oesophageal adenocarcinoma. Other studies have also reported elevated risks of Barrett's oesophagus and oesophageal adenocarcinoma in achalasia patients[27,56].

A few studies have described even larger increased risks of oesophageal cancer amongst achalasia patients. One German study reported an risk of developing oesophageal cancer up to 140 times greater in patients with achalasia than the normal population[57], which is equivalent to cancer risk in Barrett's oesophagus[58]. Furthermore, oesophageal cancer diagnosis in achalasia patients is often delayed, contributing to a poor mean survival after diagnosis of only 0.7 years[53,59]. These findings would support the need for endoscopic surveillance in achalasia patients.

However, despite the relative risk being increased, the absolute risk of cancer in patients with achalasia is still small and there would be a large number of examinations required to detect a single cancer. In fact it has been estimated that for the detection of a single cancer there would need to be 681 endoscopic examinations undertaken[53]. Despite the potential complications associated with diagnosis, treatments and increased cancer risk, achalasia patients don't experience a significant compromise to overall life expectancy[29]. The most recent guidelines indicate that surveillance endoscopy is not indicated[60].

CONCLUSION

In conclusion, achalasia remains a relatively under-researched condition with many details on aetiology, true incidence, and risk of complications still unknown. There has been some progress over the past years into the aetiology of the condition but there is a need for further research to be carried out into this field to establish causative agents. Furthermore, clarification in relation to the need for an endoscopic screening program in patients with achalasia to detect the development of oesophageal cancer is required.

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ORIGINAL ARTICLE

Molecular epidemiology and putative origin of hepatitis C virus in random volunteers from Argentina

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Abstract

AIM: To study the subtype prevalence and the phylogenetic relatedness of hepatitis C virus (HCV) sequences obtained from the Argentine general population, a large cohort of individuals was analyzed.

METHODS: Healthy Argentinian volunteers (n = 6251) from 12 provinces representing all geographical regions of the country were studied. All parents or legal guardians of individuals younger than 18 years provided informed written consent for participation. The corresponding written permission from all municipal authorities was obtained from each city or town where subjects were to be included. HCV RNA reverse transcription-polymerase chain reaction products were sequenced and phylogenetically analyzed. The 5′ untranslated region (5′UTR) was used for RNA detection and initial genotype classification. The NS5B polymerase region, encompassing nt 8262-8610, was used for subtyping.

RESULTS: An unexpectedly low prevalence of HCV infection in the general population (0.32%) was observed. Our data contrasted with previous studies that reported rates ranging from 1.5% to 2.5%, mainly performed in selected populations of blood donors or vulnerable groups. The latter values are in keeping with the prevalence reported by the 2007 Argentinian HCV Consensus (approximately 2%). HCV subtypes were

distributed as follows: 1a (25%), 1b (25%), 2c (25%), 3a (5%), and 2j (5%). Two isolates ascribed either to genotype 1 (5%) or to genotype 3 (5%) by 5'UTR phylogenetic analysis could not be subtyped. Subtype 1a sequences comprised a highly homogeneous population and clustered with United States sequences. Genotype 1b sequences represented a heterogeneous population, suggesting that this genotype might have been introduced from different sources. Most subtype 2c sequences clustered close to the 2c reported from Italy and Southern France.

CONCLUSION: HCV has a low prevalence of 0.32% in the studied general population of Argentina. The pattern of HCV introduction and transmission in Argentina appears to be a consequence of multiple events and different for each subtype.

Key words: Hepatitis C virus NS5B subtyping; Molecular epidemiology; Hepatitis C virus; Argentina; Hepatitis C virus 5' untranslated region

Core tip: This study reports an unexpectedly low prevalence of hepatitis C virus (HCV) (0.32%) in the general population of Argentina, with a subgenotype distribution of 1a (25%), 1b (25%), 2c (25%), 3a (5%), and 2j (5%) while two isolates ascribed either to genotype 1 (5%) or to genotype 3 (5%) could not be subtyped. Phylogenetic analysis of the NS5B region has enabled us to define the pattern of HCV introduction and transmission in Argentina as a consequence of multiple events that differed for each (sub)genotype studied. Furthermore, this report discusses the putative sources of HCV subgenotype introduction in Argentina.

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INTRODUCTION

The analysis of extensive sets of sequences from hepatitis C virus (HCV) isolates throughout the world has revealed the existence of six major genetic groups or genotypes (named 1 to 6), and a large number of subtypes within these groups (designated a, b, c, *etc.*)[1]. Overall sequence divergence ranges from 31% to 33% among genotypes and from 20% to 25% among subtypes[1,2]; in a single patient, cloned E1/E2 sequences may differ by up to 10%. The reason for this great variation is a high mutation rate and high level of viral replication through an error prone RNA polymerase without proofreading capacity. Consequently, in the infected individual, the virus circulates as a complex viral quasispecies[3] whose composition is subject to continuous changes due to competitive selection[4] and interactions among variants of with different levels of fitness[5]. The calculated average rate of fixation of mutations has consistently been found to range between 1.1 and 1.5 × 10^{-3} mutations per site and per year[6,7]. The rate of fixation of mutations is not evenly distributed throughout the genome, which has highly variable regions within the envelope coding genes and well conserved regions, such as the 5' untranslated region (5'UTR). The practical consequence of the high conservation at 5'UTR for HCV genotyping is that this region contains insufficient variation to solve HCV classification at the level of viral subtypes[8]. Furthermore, genotype 6 variants other than 6a and 6b show 5'UTR sequences identical or similar to those of type 1 and, therefore, cannot be differentiated[9-11]. It has been reported that sequence analysis of the highly conserved region in NS5B known as the "Okamoto region" (nt 8282 to 8610 in the H77 reference genome)[8] provides the best concordance with the full length genome phylogeny for precise genotype identification. The same primers can amplify genotypes 1 to 5, and they are less efficient for genotype 6 isolates, but they facilitate analysis and classification of the amplified sequences[11].

The prevalence of HCV infection in Argentina has been reported at 1.5% when all age groups are considered, and up to 2.0%-2.5% in adults[12], a rate in keeping with the value reported by the Argentinian Consensus on Hepatitis C (approximately 2%) in 2007. A higher prevalence (4.9%-5.7%) has been described in small rural communities[13,14]. HCV genotype distribution in Argentina in groups at risk of HCV infection (*e.g.*, transfusion patients, hemodialysis patients, intravenous drug users, healthcare workers) has been reported at 53.5% for genotype 1, 23% for genotype 2 (mainly by subtype 2c [HCV-2c]), 8.6% for genotype 3, and 14.8% for mixed infections[15]. Similar results have been reported in studies on sequences deposited in GenBank (GB) and analyses by the Los Alamos database http://hcv.lanl.gov, with few genotype distribution changes (79.5% for genotype 1, 13.9% for genotype 2, 3.9% for genotype 3, 2.4% for genotype 4), but with some differences depending on the HCV subgroup at risk studied[16-18]. Phylogenetic characterization of genotype 4 isolates from Argentina has traced an independent origin of the three sequences studied[17]. Interestingly, HCV-2c was the most prevalent subtype (58%) in the city of Córdoba (Central geographical region of the country), followed by HCV-1b (33%), and to a lesser extent by HCV-1a (11%), HCV-3a (3%) and HCV-4a (3%)[19,20].

Here, we report an unexpectedly low prevalence of HCV infection (0.32%) as measured by anti-HCV antibodies detected by using both a second generation enzyme immune assay (EIA) and a confirmatory immunoblotting, and HCV RNA detected by reverse transcription - nested polymerase chain reaction (RT-nested PCR)

Table 1 Epidemiological profile of the population studied

Province/city of residence	Total number of individuals	Male/female number	Age[1] (yr), mean ± SE
Buenos Aires/C.A.B.A.	1461	685/776[a]	33.4 ± 0.3 (11.2, 30)[d]
Catamarca	648	267/381	39.3 ± 0.5 (13.0, 38)[d]
Córdoba	1061	393/668[b]	37.1 ± 0.4 (12.5, 35)
Chaco	353	175/178[b]	40.2 ± 0.8 (14.1, 40)[d]
Chubut	172	83/89	37.4 ± 0.9 (12.1, 37)
Entre Ríos	474	225/249	38.5 ± 0.5 (11.6, 38)
Jujuy	176	105/71[b]	35.2 ± 0.8 (10.3, 34)[d]
Río Negro	329	149/180	40.1 ± 0.7 (12.8, 39)[d]
Salta	561	230/331	41.3 ± 0.5 (12.8, 41)[d]
San Luis	195	52/143[b]	41.6 ± 1.2 (16.3, 40)[d]
Santiago del Estero	375	164/211[b]	38.0 ± 0.7 (13.2, 35)
Tucumán	446	209/237	37.7 ± 0.6 (12.0, 35)
Total	6251	2738/3513	37.5 ± 0.2 (12.7, 35)

[1]Data are expressed as mean ± SE (SD, median). [a]$P < 0.05$, [b]$P < 0.01$, regarding the gender distribution (male/female ratio) within the whole population studied; [d]$P < 0.01$ regarding the mean age ± SD from the whole population studied. C.A.B.A.: Ciudad Autónoma de Buenos Aires, the national capital city.

targetting the 5'UTR HCV RNA in a cohort of random Argentinian volunteers. The genotypes detected and the putative origin of the HCV sequences are discussed based based on both their phylogenetic clustering and on such clustering relative to other Argentinian and worldwide derived sequences deposited in GB, in an attempt to trace how HCV could have been introduced in the local community here represented by the cohort studied.

MATERIALS AND METHODS

Throughout the 2000-2007 period, a total of 6251 serum samples were collected from healthy volunteers from 12 Argentinian provinces, as well as from the Ciudad Autónoma de Buenos Aires (C.A.B.A. - the capital city of the country) as follows: Buenos Aires province and C.A.B.A., $n = 1461$; Catamarca, $n = 648$; Córdoba, $n = 1061$; Chaco, $n = 353$; Chubut, $n = 172$; Entre Ríos, $n = 474$; Jujuy, $n = 176$; Río Negro, $n = 329$; Salta, $n = 561$; San Luis, $n = 195$; Santiago del Estero, $n = 375$; and Tucumán, $n = 446$ (Table 1).

Subjects included in this study [$n = 6251$; 2738 men; mean ± SE, 37.5 ± 0.2 years; mean ± SD = 37.5 ± 12.7; median age = 35 years (range 10-70 years)] were recruited as volunteers from the general population, local schools, and police stations, after being informed about the aim of the survey. All parents or legal guardians of individuals younger than 18 years provided informed written consent for participation. The corresponding written permission from all municipal authorities was obtained from each city or town where subjects were to be included.

Serological studies

The presence of anti-HCV antibodies was determined by using a second generation EIA test according to the manufacturer's recommendations (Abbott Diagnostics, North Chicago, IL, United States). Samples were further analyzed with a second generation recombinant immunoblot assay (RIBA 2.0: Chiron Corporation, Emeryville, CA, United States).

HCV-RNA detection and genotyping

Samples with serologically detectable anti-HCV antibodies were subjected to either RT-nested or RT-hemi-nested PCR amplification (see below). The 5'UTR region was used for RNA detection and initial genotype classification. The NS5B polymerase region, encompassing nt 8262-8610, was used for subtyping.

RNA extraction

RNA was extracted from 140 μL of serum by using the QIAamp Viral RNA Mini Kit (Qiagen Hilden, Germany). The measures to prevent contamination suggested by Kwok and Higuchi were strictly applied[21].

5'UTR RT-nested PCR amplification and sequencing

The 5'UTR RT-nested PCR was performed as follows. RT was carried out for 45 min at 42 ℃ (GeneAmp 2700 PCR system, Applied Biosystems, Foster City, CA, United States), using 50 U M-MLV reverse transcriptase, RNase H Minus, Point Mutant (200 U/μL Promega, Madison, WI, United States), 20 U RNase inhibitor (40 U/μL Promega, Madison, WI, United States), 10 mmol/L of each dNTP (Roche, Basel, Switzerland), 20 pmols of antisense PCR primer NR5 5'TGCTCATGGTGCACGGTCTACGAG3' and 1 × buffer from the high fidelity *Pfu* turbo DNA polymerase (Stratagene, San Diego, CA, United States) in a final volume of 20 μL. Then, 80 μL of PCR mix containing 1 × *Pfu* turbo buffer, 20 pmol of sense primer NF5 5'GTGAGGAACTACTGTCTTCACGCAG3' and 2.5 U *Pfu* turbo DNA polymerase were added to each tube. After an initial denaturation step of 2 min at 95 ℃, 5 initial cycles of 30 s at 94 ℃, 30 s at 55 ℃ and 2 min at 72 ℃ were carried out, followed by 35 cycles of 30 s at 94 ℃, 30 s at 60 ℃ and 2 min at 72 ℃, finishing with a single final step of 10 min at 72 ℃. Five microliters of the product were used for nested PCR, by using internal primers the internal primers, K80 5' AGCGTCTAGCCATGGCGT3' and K78 5'CACTCGCAAGCACCCTATCAGGCAGT3'. The nested PCR mix consisted of 1 × *Pfu* turbo buffer, 10 mmol/L of each dNTP, 20 pmol of internal primers, and 2.5 U of *Pfu* turbo DNA polymerase in a final volume of 100 μL. After a single denaturation step of 2 min at 95 ℃, we carried out 30 cycles of 30 s at 95 ℃, 30 s at 60 ℃, and 2 min at 72 ℃, and then, a final single step of 10 min at 72 ℃. The amplified products of 240 nucleotides length were analyzed by electrophoresis onto 2% agarose gels stained with ethidium bromide. PCR products were purified by using the QIAquick PCR purification kit for direct sequencing on an Abi Prism 310 Genetic analyser (Applied Biosystems).

NS5B RT-heminested-PCR amplification and sequencing

Extracted RNA was reverse transcribed using the de-

generate primer NS5B8704 5'GADGAGCADGATGTWATBAGCTC3' (nucleotide positions 8682-8704), where D = G + A + T, W = A + T and B = G + T + C, following the same conditions as for 5'UTR (see before). PCR was carried out by using the primer NS5B8256 5' TAYGAYACCMGNTGYTTTGACTC3' (nucleotide positions 8256-8278), where Y = C + T, M = A + C, and N = A + T + G + C, with an initial denaturation step of 2 min at 95 ℃, five initial cycles of 30 s at 95 ℃, 30 s at 43 ℃, and 2 min at 72 ℃, followed by 35 cycles of 30 s at 95 ℃, 30 s at 46 ℃ and 2 min at 72 ℃, and completed with a single final step of 10 min at 72 ℃. Heminested PCR was performed with an initial denaturation step of 2 min at 95 ℃, 30 cycles of 30 s at 95 ℃, 30 s at 48 ℃, and 2 min at 72 ℃, completed with a single final step of 10 min at 72 ℃ using primers NS5B8256 and NS5B8641 5'GARTAYCTGGTCATAGCNTCCGT3' (nucleotide positions 8641-8619), where R = A + G, to obtain a final product of 386 nucleotides. Purification and sequencing were performed as mentioned above.

Genotyping and subtyping-GB sequences

A GB query to Nucleotide collection (nr/nt), using the Megablast programme (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed for each of the NS5B sequences obtained in this study. The 100 GB sequences with the highest sequence similarity to each of our samples were selected. A tree was constructed by using either the Neighbor-joining or Fast Minimum Evolution algorithm from a given matrix of distances using the Jukes-Cantor method to calculate the distances. For every query, we obtained a tree that situates each sequence with the most closely related reference from the GB. The most similar sequences were downloaded for further analysis, the genotype was assigned, and a putative origin of local isolates was inferred. GB was accessed to download already published sequences of Argentine origin by searching with the words "HCV" and "Argentina" on the website: http://www.ncbi.nlm.nih.gov/sites/gquery. Published sequences obtained by several authors[15,17,22,23] were downloaded in Fasta format, and their length was then adjusted using the GeneDoc program[24].

Phylogenetic analysis

Nucleotide sequences were resized by using GeneDoc and aligned by the CLUSTALW program[25]. Sequence similarity with other sequences from Argentina and with other GB sequences was ascertained by both distance and parsimony methods. Statistical support for each node in the trees drawn by both methods was obtained by performing 100 or 1000 bootstrap replicates of the original nucleotide sequence alignment. Phylogenetic analysis was carried out with the PHYLIP package[26]. Trees were drawn by using the Treeview program, v. 1.6.5[27].

Genetic divergence analysis

Genetic divergences were calculated by using the DNASP program (version 3.53)[28] in three populations: our sequences grouped as a population, the closest GB sequences as another, and other Argentine sequences as the third one. Pairwise distances were calculated by using the MEGA3.1 program.

Statistical analysis

They were performed by using either the GraphPath Prism (version 5.0 for Windows) or the SigmaStat software. The non-paired Student's *t* test was used to analyze the statistical differences between the mean age ± SD of the whole population of the country regarding those values recorded from each Province (therefore, examining a data set from two groups), as well as for such comparison with previous studies. When such study was performed among three or more groups, the one-way analysis of variance (ANOVA) was applied. Pairwise distances were statistically compared by using the χ^2 test with Yates' correction (SigmaStat software). *P* values lower than 0.05 were considered statistically significant.

RESULTS

HCV prevalence in the general population of Argentina

A total of 6251 serum samples from a cohort of random volunteers was studied. Initially, 25 samples (0.40%) tested anti-HCV antibody positive as determined by EIA. However, 5 of the 25 samples failed to exhibit specific anti-HCV antibodies by immunoblotting analysis and also tested negative by RT-nested PCR to detect HCV RNA; hence, they were discarded for further studies.

HCV RNA was detected in serum samples from 7 out of 12 provinces. The prevalence of ongoing HCV infection as determined by RT-nested PCR from 12 of 23 provinces of Argentina, representing 73% of the country's total population, was 0.32%. The highest prevalence was detected in Buenos Aires province (0.62%; 9/1461), a geographical area inhabited by 40.52% of the total population of Argentina. In the remaining provinces studied, the prevalence ranged from 0% in Chaco, Chubut, Entre Ríos, Jujuy, and San Luis, to up to 0.53% in Santiago del Estero. Intermediate values were observed in Salta (0.18%) and Córdoba (0.19%), Río Negro (0.30%), as well as in Catamarca (0.46%) and Tucumán (0.45%).

Genotype 1 was the most prevalent, accounting for 55% (11/20) of infected individuals, 25% were subtype 1a (5/20) and 25% subtype 1b (5/20). The second in prevalence was genotype 2 accounting for 35% (7/20); most sequences were ascribed to subtype 2c (n = 5), except one that clustered much closer to subtype 2j reference sequences. Lastly, genotype 3 accounted for the remaining 10% (2/20; Table 2). One genotype 1, one genotype 2 , as well as one genotype 3 isolates (according to their initial 5'UTR genotype assignment) were excluded from further analysis because of their respective NS5B RT-heminested PCR amplification failure.

Phylogenetic analysis and genetic divergence

NS5B phylogenetic trees and divergence analysis were

Table 2 Genotype and subtype assignment of the Argentinian isolates studied by using both 5'-untranslated region and NS5B sequences

HCV genotype	HCV[+] 5'UTR sequences	Relative	From total population (n = 6251)	HCV subtype	HCV[+] NS5B sequences	Relative	From total population (n = 6251)
1	11	55.00%	0.176%	1a	5	25.00%	0.080%
				1b	5	25.00%	0.080%
				1[1]		5.00%	0.016%
2	7	35.00%	0.112%	2c	5	25.00%	0.080%
				2j	1	5.00%	0.016%
				2[1]		5.00%	0.016%
3	2	10.00%	0.032%	3a	1	5.00%	0.016%
				3[1]		5.00%	0.016%
Total	20	100.00%	0.320%	-	17	100.00%	0.320%

[1]Untypeable at NS5B due to reverse transcription-heminested polymerase chain reaction amplification failure. HCV: Hepatitis C virus; 5'UTR: 5'-untranslated region.

Table 3 Genetic divergence (DNASP software)

	Argentine general population sequences	GB deposited sequences from Argentina	Closest GB deposited sequences
HCV-1a			
Argentine general population sequences	0.034		
GB deposited sequences from Argentina	0.042	0.040	
Closest GB deposited sequences	0.034	0.037	0.032
HCV-1b			
Argentine general population sequences	0.082		
GB deposited sequences from Argentina	0.074	0.046	
Closest GB deposited sequences	0.054	0.050	0.045
HCV-2c			
Argentine general population sequences	0.087		
GenBank deposited sequences from Argentina	0.092	0.098	
Closest GB deposited sequences	0.076	0.080	0.076

Hepatitis C virus (HCV)-1b (Argentine general population sequences): 0.082 *vs* 0.054, $P < 0.001$; 0.082 *vs* 0.074, $P > 0.05$; HCV-1b [GenBank (GB) deposited sequences from Argentina]: 0.046 *vs* 0.050, $P < 0.001$; HCV-2c (Argentine general population sequences): 0.087 *vs* 0.076, $P < 0.001$; 0.087 *vs* 0.092, $P > 0.05$; HCV-2c (GB deposited sequences from Argentina): 0.098 *vs* 0.08, $P < 0.001$. All P (HCV-1a) > 0.05. Genetic divergence (DNASP software), considering three separate HCV groups: our Argentine general population (sequenced in this study), Argentinian sequences already deposited in GB, and the closest GB worldwide sequences. All values obtained were statistically paired and compared by using the χ^2 test with Yates' correction.

performed by using the five HCV-1a, five HCV-1b, and five HCV-2c sequences. However, divergence could not be ruled out with those genotypes encompassing one single representative sequence (as occurred with 2j and 3a).

As expected, local NS5B sequences clustered together with their corresponding GB references (Figure 1), but the profile differed depending on the genotype. All of our HCV-1a Argentinian sequences clustered together with strong bootstrap support (92%; Figure 2A) and exhibited a low genetic divergence (0.034; Table 3). Furthermore, genetic divergence was not statistically different in comparison with other Argentinian sequences deposited in GB or with the closest non-Argentinian GB deposited sequences included in the study, suggesting a putative common source of infection/transmission.

In the case of Argentinian HCV-1b, we observed two clusters with very low bootstrapping support (19%-25%), and one sequence distantly located regarding such clusters (Figure 2B). Genetic divergence was higher (0.082) than that observed among GB deposited Argentinian 1b sequences but the differences were not statistically significant. Similarly, HCV-2c sequences were intermingled along the tree with no particular clustering (Figure 2C) and showed a high genetic divergence (0.087). Phylogenetic analysis of Argentinian HCV-1b and HCV-2c sequences suggested a different origin of infection/transmission when compared with HCV-1a sequences.

Origin of closest GB sequences

The closest GB sequences obtained by distance (UPGMA and NJ) and parsimony methods (DNAPARS) are represented in Table 4 (trees not shown). The geographic localization of the closest GB sequences is represented in Figure 3.

All local HCV-1a sequences grouped with GB United States sequences (St. Louis, Boston, or New York areas). The results suggest a narrow source of infection and not a multifocal event, and are consistent with the low degree of divergence found in the Argentine general population, despite the fact that the subjects studied resided in three different provinces (Buenos Aires, Córdoba, and Río Negro), and from whom serum sampling was performed, several hundred of kilometers apart from each other.

HCV-1b sequences grouped with those from all over the world, including Europe (Spain, Russia), Asia (China), Africa (Madagascar, Tunisia) and North America (United States), and represented a heterogeneous population (Table 3).

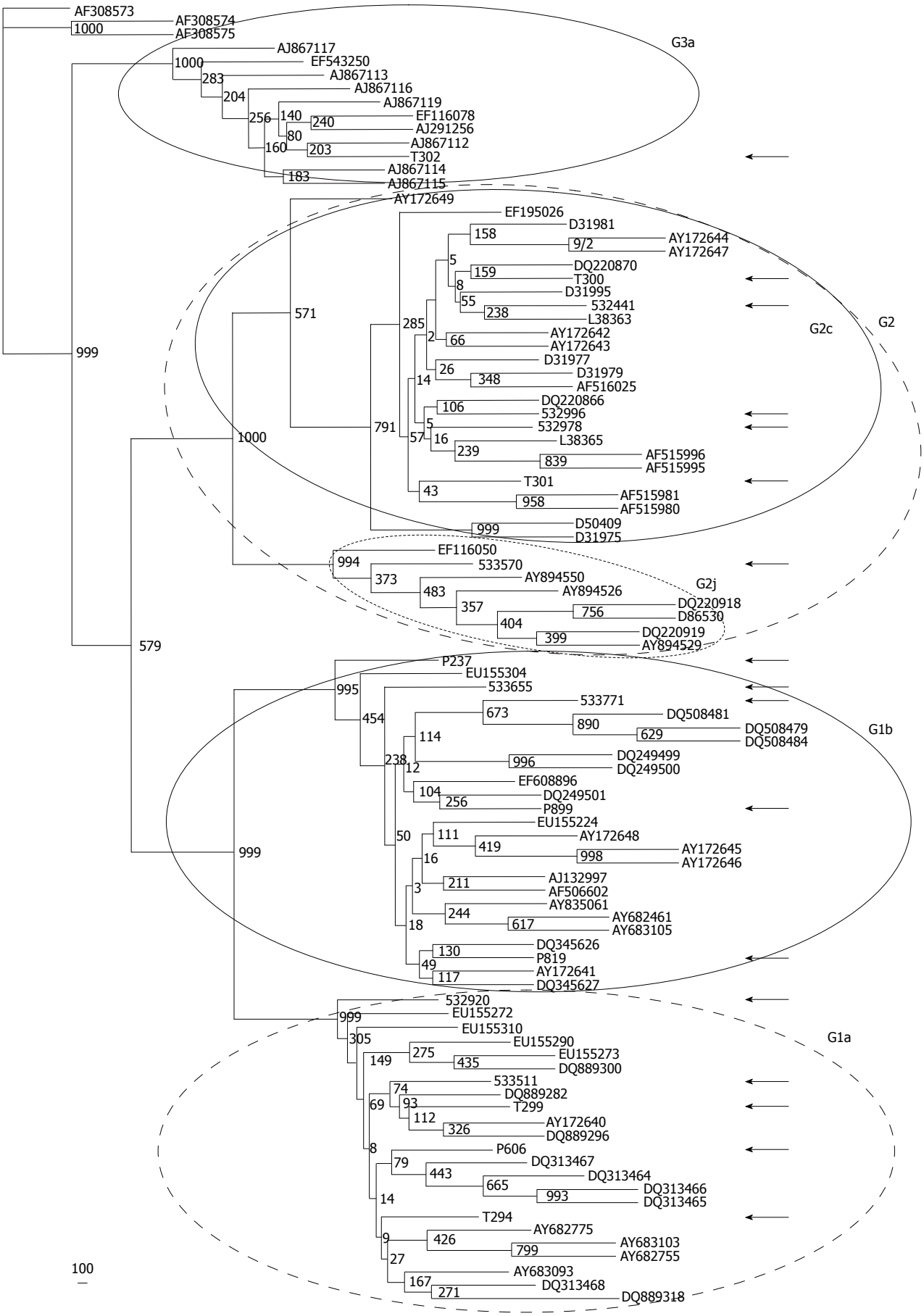
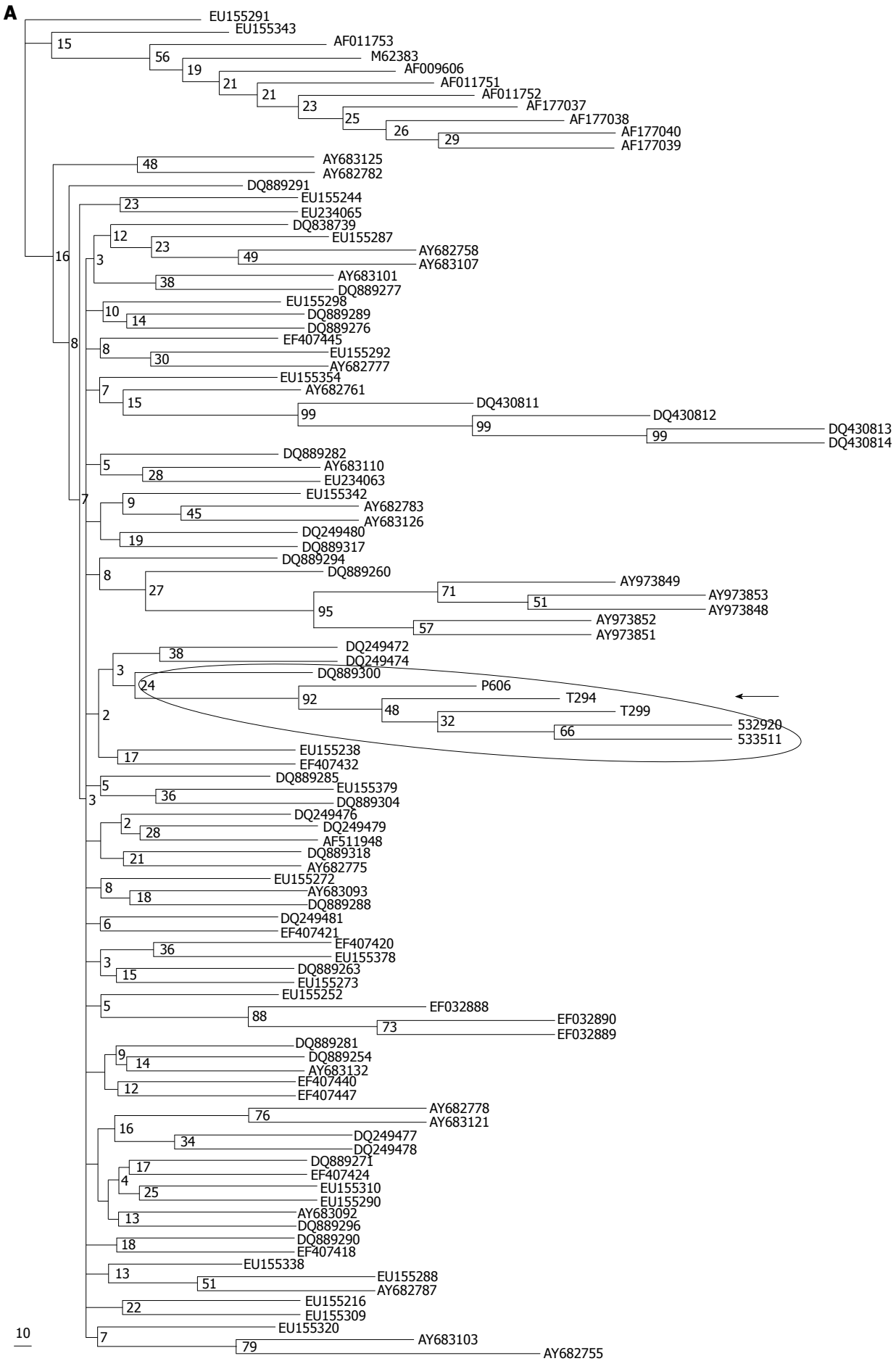


Figure 1 Assignment of Hepatitis C virus subtype isolates according to the phylogenetic tree performed by the Neighbor-Joining method, after an NS5B alignment of Hepatitis C virus sequences obtained from the 17 Argentinian volunteers studied herein, as well as from 89 reference sequences (*e.g.*, those composing subgenotype groups G1a, G1b, G2c, G2j, G3a) downloaded from GenBank. A consensus tree is shown after analyzing 1000 replicate trees. A distance scale (in nucleotide substitutions per position) is shown. Arrows indicate Hepatitis C virus sequences derived from Argentinian volunteers.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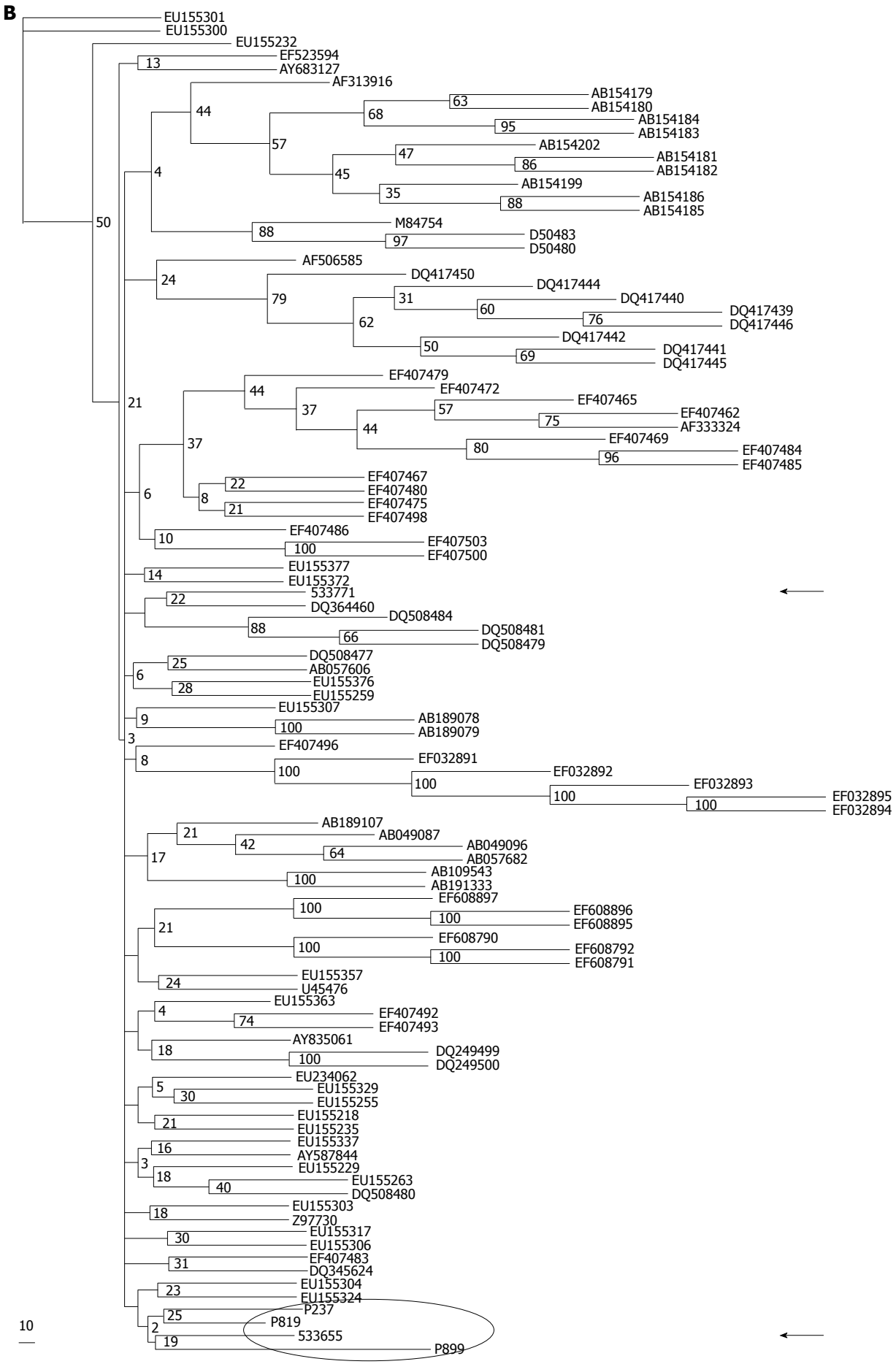
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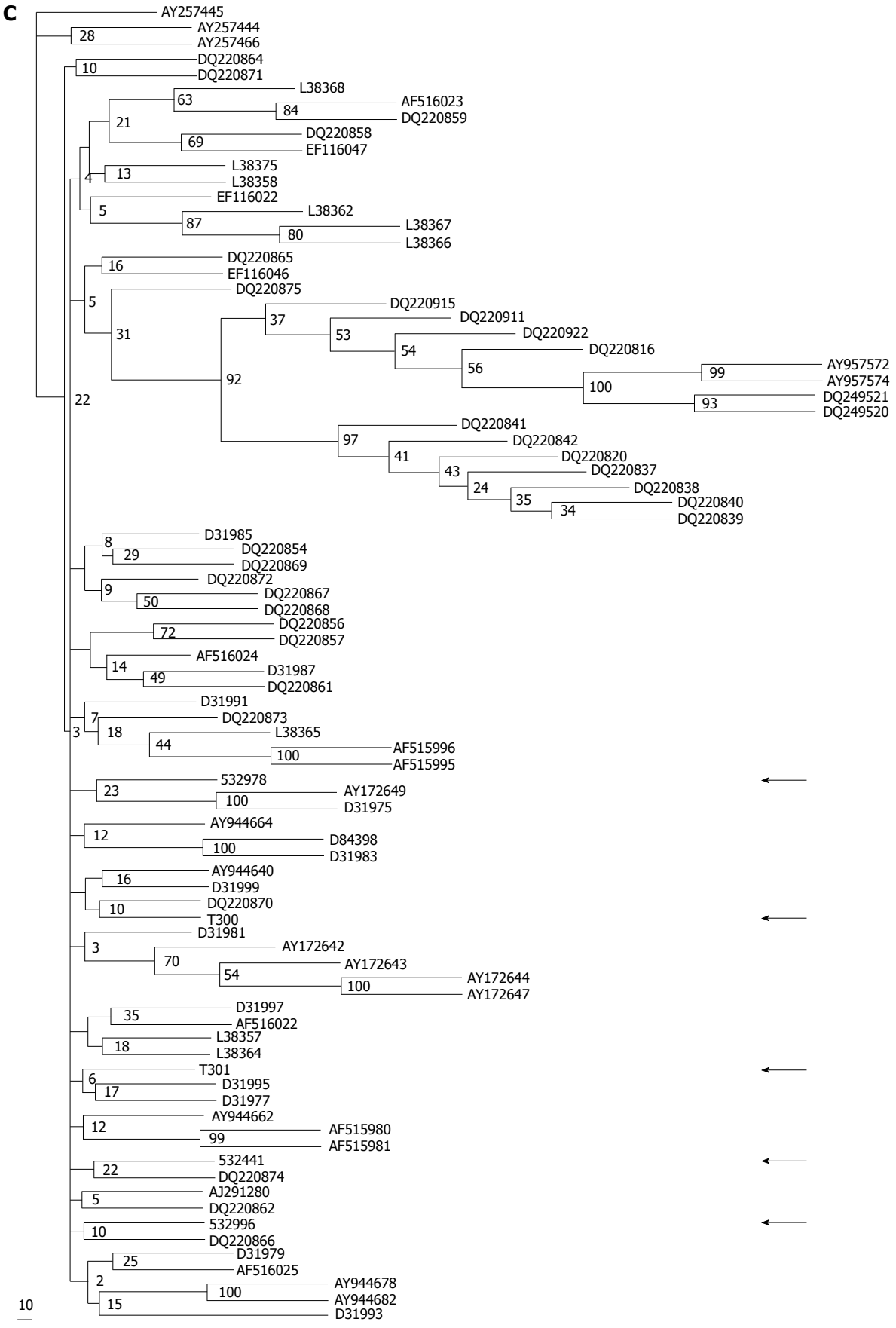

Figure 2 Phylogenetic trees performed by the Neighbor-Joining method. NS5B sequences obtained from the volunteers whose respective hepatitis C virus (HCV) isolates had been classified as HCV-1a (n = 5), HCV-1b (n = 5) or HCV-2c (n = 5) were respectively analyzed in panels A, B and C together with 100 HCV-1a, 100 HCV-1b or 81 HCV-2c sequences downloaded from the GenBank. The respective consensus trees are shown after analyzing 100 replicate trees for each HCV subtype. A distance scale (in nucleotide substitutions per position) is shown in each panel. Arrows indicate sequences obtained from Argentinian volunteers.

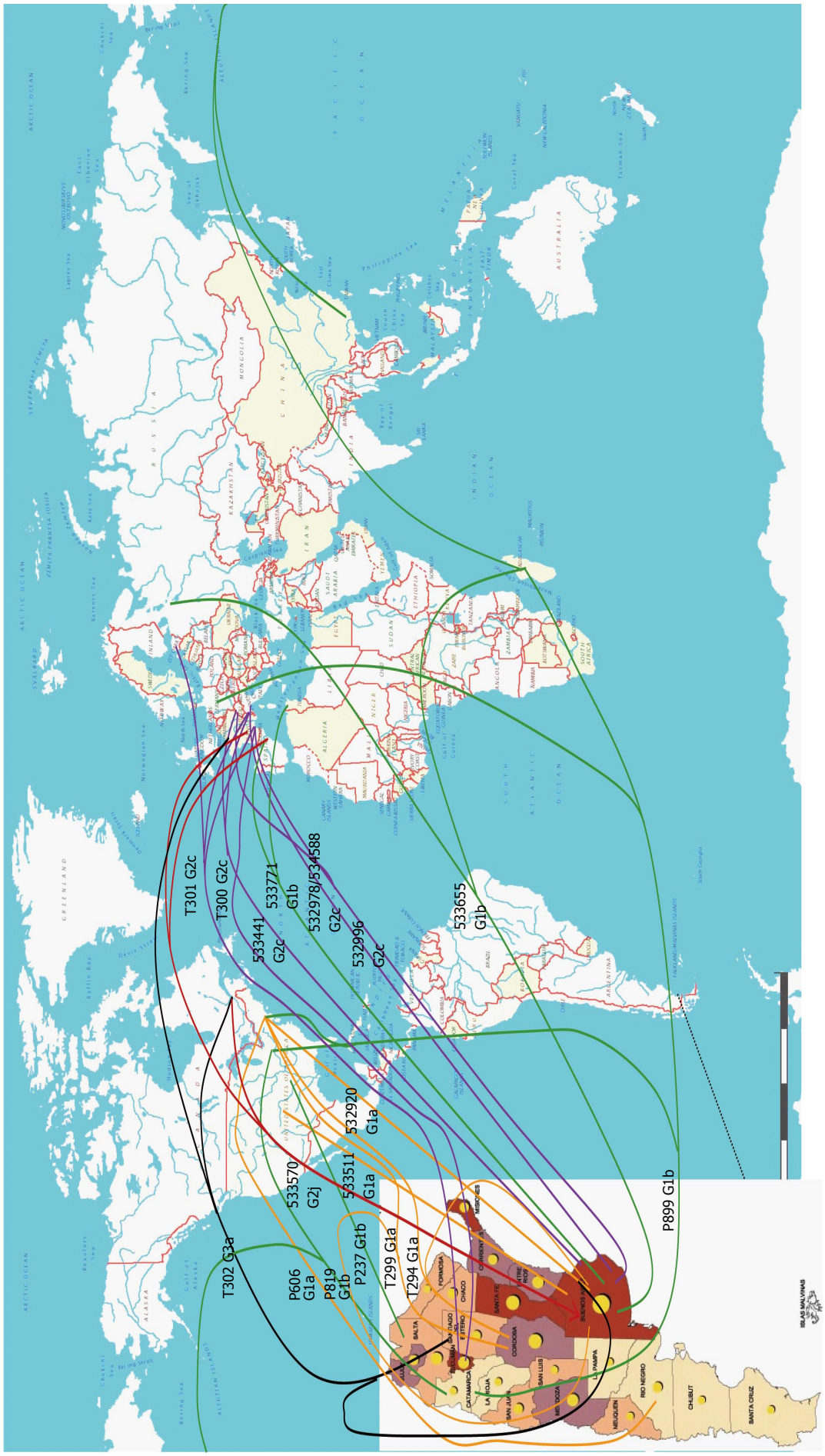

Figure 3 Each line links the Argentinian location of the sequence named on the top of the line with the world location/s of the most closely related sequence/s published in GenBank.

Table 4 Subtype assignment of each hepatitis C virus Argentinian sequence obtained in this study

Isolate	NS5B genotype	Closest GB deposited sequences	Origin
532920	1a	DQ8893001bos	United States (Massachusetts, Boston area)
533511	1a	EU155310usa	United States
		EU155290usa	
		AY683093usa	
		DQ8893001usa	
P606	1a	AY682755alb	United States (Albany, NY)
		AY683103alb	
		AY682775alb	
		DQ889318bos	United States (Massachusetts, Boston area)
		DQ889300bos	
T294	1a	DQ313467arg	Argentina
		DQ313464arg	
		DQ313465arg	
		DQ313466arg	
		DQ889300bos	United States (Massachusetts, Boston area)
T299	1a	DQ889300bos	United States (Massachusetts, Boston area)
		DQ889296bos	
		AY172640arg	Argentina
		DQ889296bos	United States (Massachusetts, Boston area)
533655	1b	AF506602rus	Russia (Western Siberia)
		DQ345627mad	Madagascar (Antananarivo)
533771	1b	DQ508484tun	Tunisia (Tunis)
		DQ508481tun	
		DQ508479tun	
		EF608896bcn	Spain (Barcelona)
P237	1b	EU155224ten	United States (Tennessee)
		EU155304ten	
P819	1b	AY835061chn	China (Foshan)
		DQ249500usa	United States (factor Ⅷ concentrate)
		DQ249499usa	
		DQ345626mad	Madagascar (Antananarivo)
P899	1b	DQ345626mad	Madagascar (Antananarivo)
		AJ132997ger	Germany
		AY682461alb	United States (Albany, NY)
		AY683105alb	
		DQ249501usa	United States (factor Ⅷ concentrate)
532441	2c	L38363swi	Switzerland
		D31981ita	Italy (France from Italians)
532996	2c	L38365swi	Switzerland
		AF15995mar	France (Marseille)
		AF515996mar	
		DQ220866tou	France (Toulouse)
532978	2c	D31975ita	Italy (France from Italians)
534588		AY172649ita	
		D50409(BEBE1)ita	
T300	2c	DQ220870fr	France (Toulouse)
T301	2c	AF516025mar	France (Marseille)
		EF195026tall	Estonia (Tallinn)
		AY944641gen	Italy (Genoa)
		D31977ita	Italy (France from Italians)
533570	2j	AY89526que	Canada (Quebec)
		AY894529que	
		AY894550que	
		EF116050que	
		DQ220919tou	France (Toulouse)
		DQ220918tou	
		D86530bcn	Spain (Barcelona)
T302	3a	EF116078que	Canada (Quebec)
		AJ291256ssd	France (Seine Saint Denis district)
		AJ867113arg	Argentina
		AJ867159arg	
		AJ867116arg	
		AJ867115arg	
		AJ867114arg	

The 3rd column from the left shows the GenBank (GB) sequences that are most similar to each of our samples, and the 4th column from the left exhibits the origin of the GB already deposited sequences.

HCV-2c sequences from the Argentine general population formed a heterogeneous group with a completely different pattern as compared with HCV-1b. The sequences clustered with GB sequences originated from HCV patients in Italy. Even the sequences reported from Southeastern France were obtained from Italians living in this region. Only one of the clustered GB sequences was documented in Estonia. Three of our 2c sequences were detected in Buenos Aires/C.A.B.A. and two in Tucumán (approximately 1200 km from C.A.B.A.).

When blasted with GB sequences, the single sequence assigned to HCV-2j clustered with those from Canada, France, and Spain, showing the high heterogeneity among the HCV-2j sequences analyzed.

Regarding the single HCV-3a local sequence, similarities were found with one sequence from Canada and another one from France, and with an additional group of five sequences reported in Argentina.

DISCUSSION

Here we report a very low prevalence of HCV infection (0.32%) in a large cohort of random volunteers from Argentina, contrasting with the 2% prevalence previously reported in studies based on selected populations in small communities, or even higher rates among highly vulnerable groups[12,29] (Hepatitis C Argentinian Consensus, 2007). The observed prevalence is lower than that reported in neighboring countries, such as Brazil (1.5%), Uruguay (1%), and Chile (0.85%)[12]. The highest prevalence was detected in Buenos Aires province and C.A.B.A., both making up the region that received the greatest number of European immigrants, especially during the first half of the 20th century (70.38% of all immigrants, 20.8% residing in C.A.B.A.; http://www.mininterior.gov.ar/poblacion/estadisticas.asp Censo2001). In this regard, recent data shows that at present most of the European immigrants from Italy and Spain are over 60-year-old (http://www.mininterior.gov.ar/provincias/archivos_prv25/6-%0Perfil_Migratorio_de_la_Argentina.pdf).

The HCV isolates studied here did not form a close national cluster separate from the GB sequences. Interestingly, genetic divergence and phylogenetic analyses showed a different profile depending on the subtype analyzed. In this sense, the HCV-1a samples, detected from subjects residing in distantly placed cities/towns (hundreds of kilometers apart from each other) from three provinces, made up a highly homogeneous population, whereas the HCV-1b and HCV-2c samples were more heterogeneous, suggesting a different profile of epidemiological origin/transmission of infection for each subtype. The high homogeneity of subtype 1a and its similarity with sequences reported from United States suggest that HCV-1a was introduced in Argentina by a common infectious source from this geographic area. This finding agrees with the model of recent HCV genotype diversification in Central and South America[30-32] compared with other continents. HCV-1b isolates formed separate clusters that were most similar to European sequences, suggesting multiple focal transmission events, likely with independent geographical origins. Interestingly, the subject whose HCV isolate showed an HCV-1b phylogenetic relationship with a Russian HCV-1b sequence stated such ethnicity. HCV-2c represents an important contribution to Argentinian HCV epidemiology (at least, 25% in this study), supporting previous observations (23%)[15]. Most of the 2c isolates clustered close to sequences reported from Italy and Southern France. In general, the 2c sequences deposited in GB represent a highly heterogeneous population with huge genetic diversity in Ghana, Guinea, Benin, and Burkina Faso in Africa[33], suggesting that HCV-2c has long been present in human populations, especially in these parts of Africa, and that it spread to Egypt, Europe and elsewhere in the 20th century[34]. It has been proposed that the introduction of HCV-2c in Italy was a consequence of close contacts between native Africans and soldiers and colonials during the colonial wars in 1882-1896 and 1911-1912[35,36]. A high prevalence of HCV-2c was observed among individuals in Italy[37-40] and Southern France, all related with Italian immigrants[41]. Coincidentally, a substantial percentage of the Buenos Aires population descends from Italian immigrants that arrived in the 20th century. Taken together, our results suggest that the introduction of HCV-2c in Argentina may have been the result of a multiple event, likely related to waves of Italian immigration. In this regard, it is worth mentioning that a high prevalence (approximately 50%) of this genotype has been reported among chronic HCV patients from Córdoba province[19,20,29], as compared with data from Buenos Aires and C.A.B.A. patients[15] and even higher rates (90%) from patients residing in Cruz del Eje, a small rural town located in the Northern region of Córdoba province, where HCV prevalence was reported to be 5%[29]. In contrast, the present study could not detect the circulation of such genotype from the general population studied in the city of Córdoba (encompassing the whole group from the homonymous province). Several hypothetical factors might have contributed to the observed discrepancy, among them, it seems worth mentioning: (1) the previously reported overall low prevalence of infection in the city of Córdoba[29] and in this study; (2) the lower contribution of HCV-2c to the total HCV genotype prevalence in such capital city located in the central region of the province, as compared with Cruz del Eje[20,29]; (3) the dissimilar nature of studied groups (patients versus general population), hence showing a dissimilar HCV infection prevalence, and consequently having lower probability to pick up HCV positive samples; and (4) the mean age ± SE of all the analyzed populations (49.77 ± 2.15 for patients from Córdoba and other locations of Córdoba Province (n = 26)[29], 66.15 ± 1.52 years for patients from Cruz del Eje (n = 49)[29], as compared with 37.1 ± 0.4 in this study (SD = 12.5; median age = 35 years; n = 668). However, the last mentioned factor failed to reach statis-

tical significance when the one way analysis of variance was carried out ($P = 0.1177$).

The recorded HCV-3a sequence exhibited similarities with isolates from France and Canada and other Argentinian isolates, in concordance with a more recent, worldwide expansion of this subtype[22]. The HCV-2j sequence showed similarities with French, Canadian, and Spanish HCV sequences. No other genotypes (4, 5 or 6) were detected in the Argentine general population studied.

In conclusion, NS5B analysis allowed an accurate classification of subtypes and enabled to perform the study of the evolution and origin of HCV infection. Here, we report a very low prevalence of HCV in the Argentine general population (0.32%). Phylogenetic analysis suggests diverse profiles of epidemiological expansion of HCV in Argentina: HCV-1a might have occurred from a putative common source, whereas HCV-1b and HCV-2c might have been introduced into the country following fluxes of immigration from other endemic areas, especially from Europe. The significantly high number of HCV-2c sequences compared to the reported data from neighboring countries may be the consequence of the high percentage of Italians migrating to Argentina from an area where such subtype was (and still is) highly prevalent. Argentina is a good example of how human practices, together with global expansion and human migration flows, have increased the HCV spread over the world. Adherence to standard universal precautions to avoid transmission should be strictly followed even in countries with a low prevalence of HCV.

ACKNOWLEDGMENTS

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COMMENTS

Background

Hepatitis C virus (HCV) is a leading cause of chronic liver disease. HCV is distributed globally, affecting all countries with an estimated worldwide prevalence of 2.3% (approximately 160 million people) of the whole general population. Comparisons of HCV nucleotide sequences derived from individuals from different geographical regions revealed the circulation of at least six major HCV genotypes and more than 50 subtypes. Accurate HCV genotyping in chronically infected patients is crucial not only for epidemiological studies but also from a clinical standpoint, since the HCV genotype orientates the treatment strategy.

Research frontiers

Direct sequencing, also referred as "population" sequencing, is the gold standard for HCV genomic sequence analysis. The viral genome region(s) sequenced must be carefully chosen, because not all of them provide accurate typing and subtyping. Since genotyping methods based on the exclusive analysis of the 5'NCR may lead to up to 10% mistyped results, there is a need to extend the analysis to coding regions such as NS5B or core. In this regard, the knowledge of the implicated HCV genotype in each patient contributes to select the appropriate treatment. Those infected with HCV genotype 1 must be treated with a triple combination of pegylated interferon-α (IFN-α), ribavirin and either telaprevir or boceprevir, whereas patients infected with other genotypes must still be treated with pegylated IFN-α and ribavirin alone. Moreover, HCV genotyping based on phylogenetic analysis, and - in case a representative sampling of a given (sub)genotype is obtained from an area - Monte Carlo Markov Chains Bayesian coalescent analysis may respectively lead to trace the origin and if such condition is met - the putative date of the Most Recent Common Ancestor of sequences.

Innovations and breakthroughs

This is a molecular epidemiological study performed in a large cohort of the local general population from 12 out of 23 Argentine provinces, as well as from the Autonomous city of Buenos Aires (the national capital). Unexpectedly, it shows a low prevalence of HCV (about 0.32%) in a general population cohort which included 6251 individuals. This low prevalence suggests that HCV could have been "recently" introduced in Argentina, as proposed by coalescent studies performed in restricted local areas of this country by other authors, where a predominant (sub)genotype was found, allowing such analysis. HCV subtypes were distributed as follows: 1a (25%), 1b (25%), 2c (25%), 3a (5%), and 2j (5%). HCV-1a sequences comprised a highly homogeneous population and clustered with United States sequences. HCV-1b sequences represented a heterogeneous population, suggesting that this genotype might have been introduced from different sources. Most HCV-2c sequences clustered close to the 2c reported from Italy and Southern France. Phylogenetic analysis is used by the authors to trace the putative source of HCV transmission and suggests that introduction of local HCV in this country is a consequence of multiple events that differed for each subtype studied. Diverse epidemiological patterns of HCV spread in Argentina might have occurred.

Applications

These new data could be useful to implement suitable measures regarding HCV surveillance by Argentine Public Health authorities.

Terminology

HCV genotype: group of HCV variants assigned to a given genetic groups (1-6) which differs from others by 31%-33% at the nucleotide level. HCV subtype (sub-genotype): group of more closely related HCV variants assigned to a given genetic group which differs from others by 20%-25% at the nucleotide level (named with lower case letters: *i.e.*, a, b, c, *etc.*).

Peer review

This is a very well done and written molecular epidemiological study which considers the investigation of the prevalence of HCV infection and subtype frequencies among adults in Argentina. It should be underlined that authors have investigated a large amount of general population from 12 provinces representing all the geographical regions of the country.

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ORIGINAL ARTICLE

Aberrant TGF-β1 signaling contributes to the development of primary biliary cirrhosis in murine model

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Abstract

AIM: To investigate whether transforming growth factor-β1 (TGF-β1) signaling pathway is involved in the pathogenesis of primary biliary cirrhosis (PBC).

METHODS: A murine model of PBC was developed by injection of polyinosinic polycytidylic acids (poly Ⅰ: C) in C57BL/6 mice, and the liver expressions of TGF β1, TGF-β receptor Ⅰ (TβR Ⅰ), TGF-β receptor Ⅱ (TβR Ⅱ), p-Smad2/3, monoclonal α-smooth muscle actin antibody (α-SMA) and α1 (Ⅰ) collagen in the mouse model and control mice were evaluated by immunohistochemistry, immunoblotting and real-time polymerase chain reaction (RT-PCR). Lymphocyte subsets in liver were analyzed using flow cytometry.

RESULTS: The mouse model had several key phenotypic features of human PBC, including elevated levels of alkaline phosphatase, antimitochondrial antibodies, portal bile ducts inflammation, and progressive collagen deposition. Compared with control mice, protein and mRNA levels of TGF β1, TβR Ⅰ, TβR Ⅱ, p-Smad2/3, α-SMA and α1 (Ⅰ) collagen in liver (1.7 ± 0.4 *vs* 8.9 ± 1.8, 0.8 ± 0.2 *vs* 5.1 ± 1.5, 0.6 ± 0.01 *vs* 5.1 ± 0.1, 0.6 ± 0.3 *vs* 2.0 ± 0.3, 0.9 ± 0.4 *vs* 3.4 ± 0.6, 0.8 ± 0.4 *vs* 1.7 ± 0.3, 1.1 ± 1.2 *vs* 11.8 ± 0.6, $P < 0.05$), and the total number and percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ and $CD8^+$ lymphocytes (0.01 ± 0.001 *vs* 0.004 ± 0.00, 0.12 ± 0.04 *vs* 0.52 ± 0.23, $P < 0.01$) were higher in the mouse model.

CONCLUSION: TGFβ1 might play a dual role in the development of PBC: it suppresses inflammatory response but operates to enhance fibrogenesis. The aberrant activity of TGF-β1 signaling contributes to the development of PBC.

Key words: Primary biliary cirrhosis; Transforming growth factor-β1; Regulatory T cell; Liver

Core tip: Primary biliary cirrhosis (PBC) is an autoimmune liver disease. Recent studies suggest that transforming growth factor-β1 (TGF-β1) signaling pathway might play an important role in the pathogenesis of PBC. However, whether TGF-β1 signaling pathway is involved in the development of PBC is still unknown. The studies have provided new data of TGF-β1 signaling pathway involving the pathogenesis of PBC, which will pose significant impact on our understanding of the pathogeneses of PBC. TGF-β1 signaling pathway is a potential target for PBC treatment.

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a progressive autoimmune liver disease characterized by portal inflammation and immune-mediated destruction of the intrahepatic bile ducts. Damage of bile ducts is associated with cholestasis, and eventually leads to liver failure[1].

Cytokines are involved in cell-to-cell interaction *via* specific receptors, inflammatory response amplification, immune regulation and fibrogenesis. Transforming growth factor-β1 (TGF-β1) is a prominent antiproliferative and profibrogenic cytokine that signals through TGF-β receptor Ⅱ (TβRⅡ), and receptor I (TβR I), that in turn phosphorylate Smads at the mad homology 2 domain[2]. Perturbation of TGF-β1 signaling has been implicated in several developmental disorders and in various human diseases including cancer, fibrosis and autoimmune disease[3-5]. Mice transgenic of a dominant negative form of TβRⅡ, under the CD4 promoter lacking the CD8 silencer[6], spontaneously developed features characteristic of PBC[7]. A compromised cytoarchitecture and polarized trafficking of TGF-β1 signaling molecules including embryonic liver fodrin and Smad3 were also noted in the pathogenesis of PBC[8]. Moreover, TGF-β1 was a marker for fibrosis and reflected severity of disease in patients with PBC[9,10]. Therefore, aberrant TGF-β1 signaling contributes to a loss of self tolerance to autoantigens in the liver, which in turn leads to autoimmunity.

We developed an animal model of PBC by polyinosinic polycytidylic acids (poly I : C) injection in genetically susceptible C57BL/6 female mice that would allow the analysis of the early cellular events of PBC[11,12]. We found that TGFβ1 played a dual role in the development of PBC: it suppressed inflammatory response but operated to enhance fibrogenesis. The aberrant TGF-β1 signaling contributed to the development of PBC.

MATERIALS AND METHODS

PBC animal model

Adult 6-8 wk-old C57BL/6J (H-2b) mice were purchased from Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC, Beijing, China). They were maintained separately at the Department of Laboratory Animal, Peking Union Medical College Hospital (PUMCH), China, under controlled conditions (22 ℃, 55% humidity, and 12 h day/night). All animals received adequate care according to good laboratory practice guidelines. The study protocol was approved by Committee of Animal Experimentation, PUMCH and CAMS. Female C57BL/6 mice were injected with 5 mg/kg poly I : C (Invivogen Co. San Diego, United States) or normal saline (NS) as controls twice a week for 24 consecutive weeks, according to the protocol of Okada[11].

At weeks 8 and 24, six mice of each group were sacrificed by cervical dislocation. Livers were fixed in buffered formalin (10%). Sera and tissue specimens were stored at -80 ℃. The serum levels of alkaline phosphatase (ALP) and alanine amino-transferase (ALT) were measured by commercially available kit (WAKO Pure Chemical Industry, Osaka, Japan) exactly according to the manufacturer's protocol.

Antimitochondrial antibodies detection

Antimitochondrial antibodies (AMA) and M2 were detected by the commercial immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA) kits (EUROIMMUN, Germany) and immunoblotting kits (IMTEC Corporation, Germany), according to the manufacturer's protocol. Fluorescein isothiocyanate (FITC) or horseradish peroxidase (HRP)-conjugated monoclonal goat anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) was used as the secondary antibody. Plates were read at 450 nm with a microplate reader (Bio-RAD Model 550, Tokyo, Japan). Sera with optical density (OD) values greater than the mean ± 2SD from the negative controls were regarded as AMA positive.

Histopathology

Formalin-fixed, paraffin-embedded tissue sections were cut into 5 μm slices for routine hematoxylin and eosin staining. Tissues were also stained with Azan to detect collagen deposition[13]. Briefly, sections were deparaffinized in xylene, dehydrated, rehydrated in distilled water, immersed in 5% potassium dichromate solution for 30 min, and stained with azocarmine G for 30 min. Sections were then immersed in 3% 12 tungsto (Ⅳ) phosphoric acid n-hydrate solution for 1 h and stained with aniline blue-orange G for 20 min.

Immunohistochemistry

Antibodies against CD4 (1/200; L3T4, eBioscience) and CD8 (1/100; 53-6.7; Biolegend) were used for immunohistochemical staining of the portal tract infiltrates. Anticytokeratin 7 (1/50; RCK 105; BD Bioscience, San Jose, CA, United States) was used to detect biliary cell. Antibodies against TGF-β1 (1/200; V), TβR I (1/200; T-19), TβRⅡ (1/200; C-16), p-Smad2/3 (1:50; Ser 433/435) (all obtained from Santa Cruz Biotechnology, Dallas, Texas, United States) and monoclonal α-smooth muscle actin antibody (α-SMA, 1:250; 1-4A; Sigma, St. Louis, MO, United States) were used to detect the expressions of TGF-β1 signaling proteins. Briefly, after deparaffinization, sections were incubated in a Decloaking Chamber (Biocare Medical, CA, United States) set point: SP1 123 ℃ for 2 min, SP2 85 ℃ for 10 s, SP limit 10 ℃), soaked in 3% H_2O_2 methanol solution for 5 min, then 15 min in 1 × Universal blocking solution (Bio-Genex, CA, United States) and

Table 1 Primers for real-time polymerase chain reaction

Gene	Genbank no	Forward primer (5' to 3')	Reverse primer (5' to 3')
TGFβ1	NM_011577	TGCTAATGGTGGACCGCAA	CACTGCTTCCCGAATGTCTGA
TβR I	NM_009370	ATGGTTCCGAGAGGCAGAGAT	CCATGTCCCATTGTCTTTGTTG
TβR II	NM_009371	CCAGAAGTCCTGCATGAGCAA	TGGCAAACCGTCTCCAGAGTA
Smad2	NM_010754	TCTCCGGCTGAACTGTCTCCTA	GCGATTGAACACCAGAATGCA
Smad3	NM_016769	ATGGAGCTCTGTGAGTTTGCCT	TGGAGGTAGAACTGGCGTCTCT
α-SMA	NM_007392	CTATTCAGGCTGTGCTGTCCCT	GCCCTCATAGATAGGCACGTTG
α1(I) collagen	NM_007742	CCCAAGGAAAAGAAGCACGTC	AGGTCAGCTGGATAGCGACATC
GAPDH	NM_008084	AGCCTCGTCCCGTAGACAAAA	TGGCAACAATCTCCACTTTGC

TGF: Transforming growth factor; TβR: TGF-β receptor; SMA: Smooth muscle actin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

20 min in 10% goat serum to prevent nonspecific staining. After that, sections were incubated with primary antibodies for 1 h at room temperature in a moist chamber. After three washes with 0.1% Tween 20 in PBS (PBST) for 5 min, Envision™ (DakoCytomation, Glostrup, Denmark) was applied according to the procedure and counterstained with Mayer's hematoxylin (DakoCytomation) or DAPI (4',6-diamidino-2-phenylindole 2HCI, D9542, Sigma).

Western blotting

Liver tissue was homogenized in an Ultra-Turrax homogenizer in RIPA buffer containing 1 mmol/L PMSF and protease inhibitors. After high-speed (12700 *g*) centrifugation at 4 ℃, the protein in the supernatant was separated by 10% SDS-PAGE (20 μg per lane), and transferred onto a PVDF membrane. After blocking with 1.5% bovine serum albumin (BSA) in Tris-buffered saline, TGF-β1, TβR Ⅰ, TβR Ⅱ, p-Smad2/3, α-SMA and α1 (Ⅰ) collagen were detected using rabbit polyclonal antibodies against TGF-β1 (1:1000), TβR Ⅰ (1:1000), TβR Ⅱ (1:1000), p-Smad2/3 (1:2000), α1 (Ⅰ) collagen (1:2000), and α-SMA (1:400), respectively, and then incubated with anti-rabbit and mouse IgG HRP conjugated (Promega, Madison, WI, United States). Specific binding was detected using the Super Signal West Dura Extended Duration Substrate (PIERCE, Rockford, IL, United States) with a FluorTech 8800 gel doc system (Alpha Innotech, CA, United States) equipped with a chemiluminescent filter.

Real-time PCR

Total RNA was isolated using TRI-Reagent (Sigma). Real-time PCR was carried out as described[14]. DNase Ⅰ-treated total RNA (1 μg) was used for synthesis of the first strand of cDNA. Reverse transcription conditions were as follows: 42 ℃ for 15 min, 95 ℃ for 5 min and 5 ℃ for 5 min (one cycle). Real-time PCR was carried out in 25 μL of reaction solution (2.5 μL of 10 × buffer, 5 mmol/L of each dNTP, 10 mmol/L $MgCl_2$, 200 nmol/L primers and 0.75 unit of platinum® Taq polymerase; all from Invitrogen) plus 1 μL of SYBR Green (1:2000; BioWhittaker, Richland, ME, United States). No genomic DNA contamination or pseudogenes were detected by PCR in the absence of the reverse transcription step in the total RNA used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an invariant control. The reactions started at 95 ℃ for 7 min, followed by 40 cycles of 95 ℃ for 20 s, 54 ℃ for 30 s and 72 ℃ for 30 s. Melting peaks of PCR products were determined by heat denaturation from 60 to 95 ℃ at 0.2 ℃/s. Fold changes in the mRNA levels of target genes relative to the endogenous GAPDH control were calculated as suggested by Schmittgen *et al*[15]. Table 1 lists the primers used in real-time PCR.

Real-time PCR was performed for quantitative analyses according to standard protocol using the SYBR Green PCR Master Mix and ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Tokyo, Japan).

Flow cytometry

Livers were first perfused with PBS containing 0.2% BSA, passed through a nylon mesh, and resuspended in PBS/0.2% BSA (EMD chemicals, Gibbstown, NJ, United States). Hepatocytes were removed as pellets after centrifugation at 700 r/min for 60 s periods[16]. Lymphocytes from suspended liver cells were then isolated using Histopaque-1077 (Sigma Chemical Co. St. Louis, MO, United States). After centrifugation, cells were washed with PBS/0.2% BSA, and the viability of cells confirmed by trypan blue dye (Sigma Chemical Co. St. Louis, MO, United States) exclusion. Cell preparations were then pre-incubated with anti-mouse FcR blocking reagent and then incubated at 4 ℃ with a combination of fluorochrome-conjugated antibodies, including anti-CD4 FITC, anti-CD25 APC, anti-CD8 PECy5, anti-Foxp3 PE (all from eBioscience CA, United States). Multiple-color flow analyses were performed using a FACScan flow cytometer upregulated by Cytec Development (Fremont, CA, United States) to allow for 4-color analysis. Acquired data were analyzed with CELLQUEST Software (BD Biosciences CA, United States) and FlowJo Software (Tree star, Inc., Ashland, OR, United States).

Statistical analysis

Results are expressed as mean ± SD and were evaluated using Mann-Whitney *U* tests for comparison between samples from mouse model and littermates, with $P < 0.05$ considered significant.

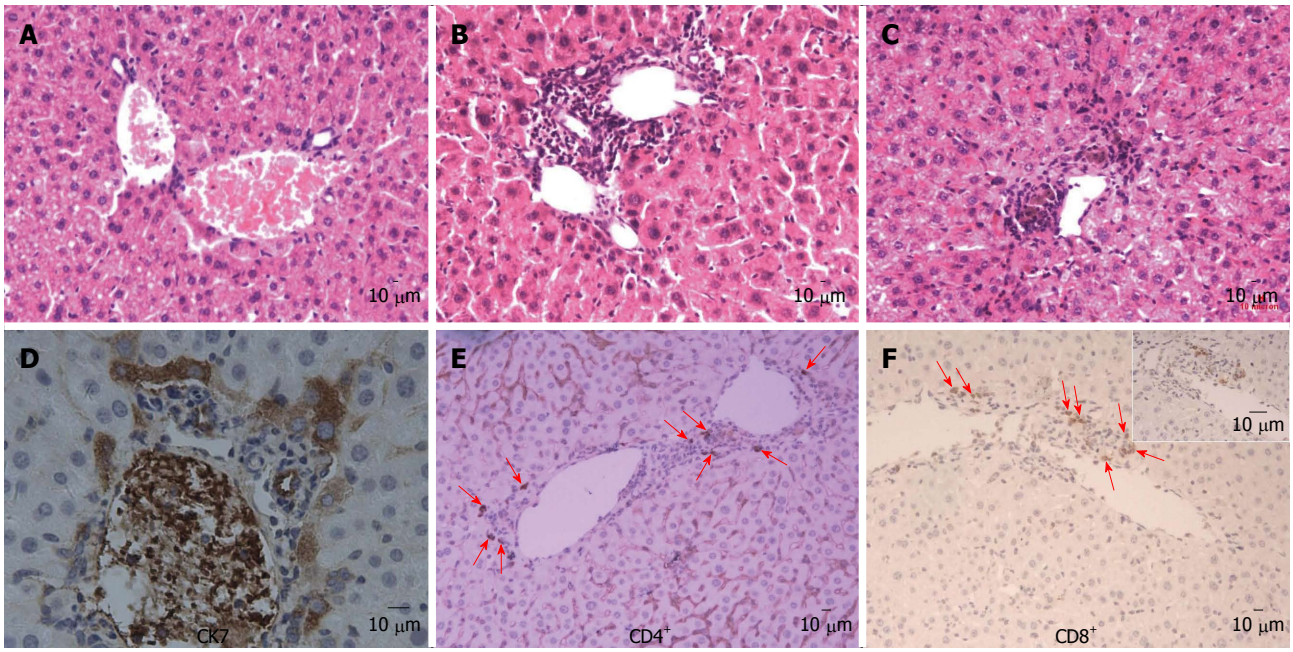

Figure 1 Histological features of the liver. A: Control mice; B-F: Mice model; B: Lymphocytic infiltration (red arrows) around the small bile ducts within the portal tracts at week 8; C: Bile plugs were seen in canaliculi at week 24; D: CK-7 expression in periportal proliferated bile ductile and intralobular hepatocytes; E: $CD4^+$ lymphocytes infiltration; F: $CD8^+$ lymphocytes infiltration (bar 10 μm).

RESULTS

Histological features in Poly I : C induced animal model

The serum levels of ALT, ALP and total bilirubin in the mouse model were higher than in the control mice (105.5 ± 36.9 IU/L *vs* 28.2 ± 2.9 IU/L, $P = 0.006$; 138.2 ± 15.3 IU/L *vs* 74.8 ± l8.5 IU/L, $P = 0.025$; and 2.8 ± 0.4 mg/dL *vs* 0.95 ± 0.12 mg/dL, $P = 0.043$). Mouse model displayed an increase AMA titer over time. By week 24, serum samples of the six mouse models were all positive for AMA/M_2. In the mouse model, the mean titer of anti-M_2 was significantly higher at week 24 than at week 8 ($P < 0.0005$), while in the control mice AMA/M_2 was not detected. The time table of AMA in the mouse model resembled that in human PBC, of which the disease is not observed in childhood and typically develops in the fourth or fifth decade of life.

In the liver of the mouse model, moderate to severe infiltration of lymphoid cells was detected within the portal tracts in association with bile duct damage and a mild interface hepatitis (piecemeal necrosis) at week 8 (Figure 1B) and bile plugs were seen in canaliculi around portal tracts at week 24 (Figure 1C), which was absent in control mice (Figure 1A). Direct bile duct destruction was determined by the detection of scattered portal infiltration of CK-7 positive cells. Moreover, in liver tissues from some mice models, biliary cell destruction was so severe that identification of an intact bile duct structure was impossible and all biliary-type and hepatocytes were CK-7 positive, particularly in samples with cholestasis (Figure 1D). Immunohistochemical analysis demonstrated infiltration of $CD4^+$ and $CD8^+$ lymphocytes around small bile ducts that were absent in control mice (Figure 1E and F).

In situ detection of TGF β1, TβR Ⅰ, TβR Ⅱ, p-Smad2/3, α-SMA and α1 (Ⅰ) collagen in liver

In mouse model, expression of TGF β1 in periportal and intralobular regions became more prominent over time (Figure 2A-D). At week 8, there were positive expressions of TβR Ⅰ and TβR Ⅱ in some periportal hepatocytes and biliary ductile endothelial cells (Figure 2E-H). At week 24, distribution of TβR Ⅰ and TβR Ⅱ became more extensive and prominent (Figure 2F and I).

In mouse model, intranuclear staining of p-Smad2/3 was observed in some periportal and intralobular hepatocytes at week 8, and became more prominent at week 24 (Figure 3A-C), α-SMA positive staining and collagen deposition around portal areas were observed at week 8 (Figure 3E and H), and extension into surrounding parenchyma at week 24 (Figure 3F and I), which was absent in the liver of control mice (Figure 3D and G).

Immunoblot of TGF β1, TβR Ⅰ, TβR Ⅱ, p-Smad2/3, α-SMA and α1 (Ⅰ) collagen

Immunoblot analysis of TGF-β1, TβR Ⅰ, TβR Ⅱ, p-Smad2/3, α-SMA and α1 (Ⅰ) collagen of the liver homogenates from mouse model and control mice at week 8 and 24 is shown in Figure 4. Compared with that from control mice, there were increasing expressions of TGF-β1, TβR Ⅰ, TβR Ⅱ, p-Smad2/3, α-SMA and α1 (Ⅰ) collagen of the liver homogenates from mouse model as time increased.

Real-time PCR of TGF β1, TβR Ⅰ, TβR Ⅱ, Smad2/3, α-SMA and α1 (Ⅰ) collagen

As shown in Table 2, the mRNA levels of TGF-β1, TβR Ⅰ, TβR Ⅱ, Smad2, Smad3, α-SMA and α1 (Ⅰ) collagen

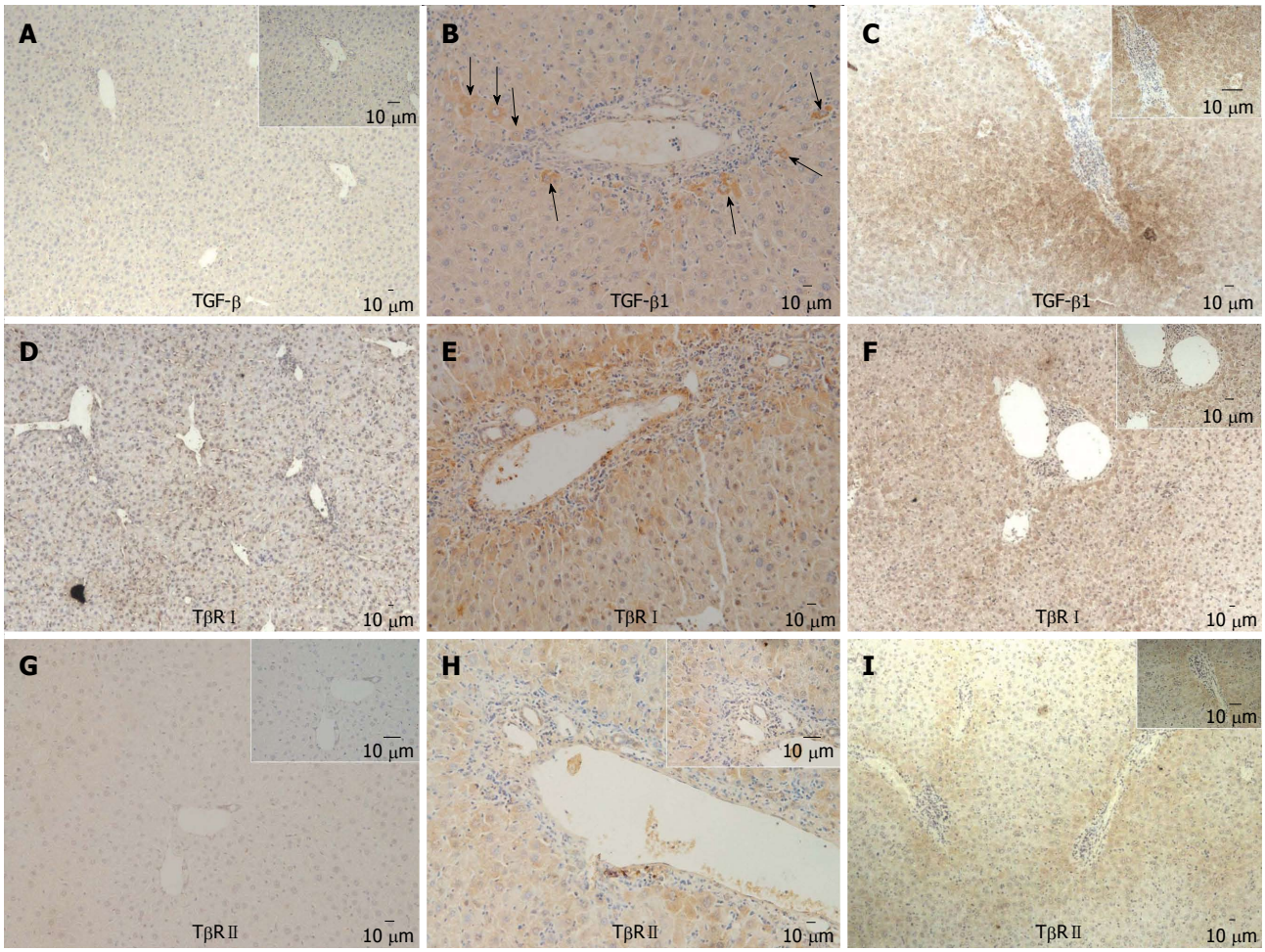

Figure 2 Expressions of transforming growth factor-β1, transforming growth factor-β receptor Ⅰ, transforming growth factor-β receptor Ⅱ in liver. A, D and G: Control mice; B, C, E, F, H and I: Mouse model; A-C: Transforming growth factor-β1 (TGF-β) expression; D-F: TGF-β receptor Ⅰ (TβR Ⅰ) expression; G-I: Transforming growth factor-β receptor Ⅱ expression (bar 10 μm).

Table 2 mRNA levels of transforming growth factor-β1 transforming growth factor-β receptor Ⅰ, transforming growth factor-β receptor Ⅱ, Smad2, Smad3, α-smooth muscle actin and α1 (Ⅰ) collagen in mouse model and control mice

	Control mice	Mouse model	
		week 8	week 24
TGF-β1	1.7 ± 0.4	7.0 ± 1.8[b]	8.9 ± 1.8[b]
TβR Ⅰ	0.8 ± 0.2	2.8 ± 0.7[b]	5.1 ± 1.5[b]
TβR Ⅱ	0.6 ± 0.01	1.9 ± 0.9[b]	5.1 ± 0.1[b]
Smad2	0.6 ± 0.3	3.8 ± 1.1[b]	2.0 ± 0.3[b]
Smad3	0.9 ± 0.4	1.7 ± 0.8[a]	3.4 ± 0.6[b]
α-SMA	0.8 ± 0.4	1.8 ± 0.1[a]	1.7 ± 0.3[b]
α1 (Ⅰ) collagen	1.1 ± 1.2	11.0 ± 1.5[b]	11.8 ± 0.6[b]

The mRNA fold changes were calculated using glyceraldehyde-3-phosphate dehydrogenase as a control. Values were expressed as mean ± SD from 3 independent experiments. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control mice. TGF: Transforming growth factor; TβR: TGF-β receptor; SMA: Smooth muscle actin.

of liver homogenates from mouse model at weeks 8 and 24 were higher than that from control mice.

Flow cytometric analysis of lymphocyte subsets in liver

After poly I : C injection, the total numbers of lymphocytes significantly increased in the liver of mouse model (Table 3). Although the total number of intrahepatic $CD4^+$ lymphocytes increased, the percentage of $CD4^+$ cells in the lymphocytes did not (Figure 5). In contrast, the $CD8^+$ population in mouse model significantly increased in both total number and percentage compared with that in controls (Figure 5). In addition, the mouse model had a marked increase in the number as well as percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ lymphocytes compared with control mice (Table 3 and Figure 5). This finding is particularly interesting, as previous studies reported a decrease in precursors of $CD4^+$ $CD25^+$ regulatory T cells (Treg) in the peripheral blood of PBC patients[7,17,18], and several recent reports demonstrated increased infiltration of $FOXP3^+$ Treg in damaged organ or target tissues in autoimmune diseases[19-21].

DISCUSSION

Our study demonstrated that this mouse model mimic several key phenotypic features of human PBC. It had elevated levels of ALP, AMA, portal bile ducts inflammation, and progressive collagen deposition. In human PBC, there is a ten-fold increase in frequency of $CD8^+$

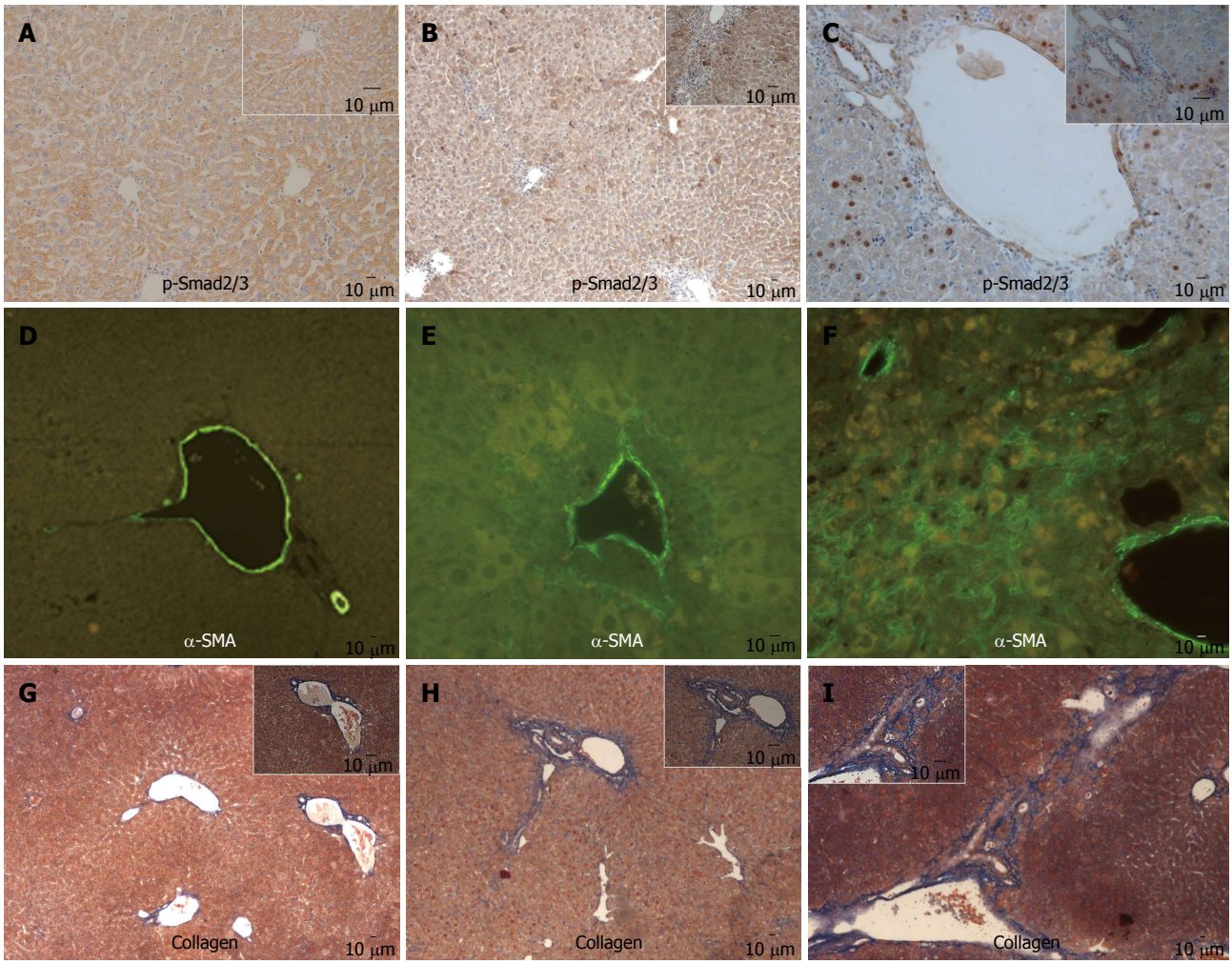

Figure 3 Expression of p-Smad2/3, α-smooth muscle actin antibody and collagen in liver. A, D and G: Control mice; B, C, E, F, H and I: Mouse model. A-C: p-Smad2/3 expression; D-F: α-smooth muscle actin (α-SMA) antibody expression; G-I: Collagen expression (bar 10 μm).

Table 3 Phenotype of mononuclear cells in the liver

	Mouse model	Control mice
Total cell number (× 10^6)	1.6 ± 0.47[a]	0.48 ± 0.32
$CD4^+$ (× 10^6)	0.06 ± 0.01	0.04 ± 0.01
$CD8^+$ (× 10^6)	0.58 ± 0.11[a]	0.08 ± 0.03
$CD4^+/CD8^+$	0.12 ± 0.04[a]	0.52 ± 0.23
$CD4^+$ $CD25^+$ $FOXP3^+$ (× 10^6)	0.01 ± 0.001[b]	0.004 ± 0.001

[a]$P < 0.05$, [b]$P < 0.01$ *vs* control mice.

T cells specific for PDC-E2 in liver compared with that in peripheral blood, and it correlates with biliary ductular damage[18,22,23]. Interestingly, our mouse model also had increased $CD8^+$ lymphocyte infiltration in liver, which is consistent with the chronic autoimmune nature of the disease. CK-7 is regarded as a histological marker for progression in PBC and indicates poor prognosis[24]. Hepatocytes do not normally express CK-7 except in the advanced stage of PBC, which was also observed in our study. Taken together, this animal model had several key phenotypic features and would allow us to analyze the early cellular events of PBC.

TGF-β1 is the key regulator in the pathogenesis of hepatic fibrosis, and appears to aggregate in the liver of PBC patients[25,26]. The selective abnormality of the TGF-β1 signaling pathway in T lymphocytes leads to impairment to peripheral tolerance and spontaneously development of features characteristic of PBC[7]. TGF-β1 is an essential modulator of Foxp3 expression in Tregs cells[20], conditioning their suppressive function. Recent studies have demonstrated reduction in the number of circulating Tregs in patients with PBC[21]. In addition, it is reported that the population of Tregs coexpressing Foxp3 and TGF-β1 decreases with age in female NOD mice[27]. Tregs produce elevated levels of TGF-β1, and the fact that TGF-β1 signaling receptors are up-regulated on the membrane of Tregs, underscores the potential for autocrine and/or paracrine receptor-ligand interaction in these cells. TGF-β1 is a positive regulator of Tregs expansion and inhibits autoimmune diseases *via* regulation of the size of Tregs pool *in vivo*[28]. Our study found elevated levels of TGF-β1 as well as the total number of $CD4^+$ $CD25^+$ $FOXP3^+$ Treg in the liver of mouse model, which seems different from some studies[29-31]. However, there were also several reports demonstrating increased

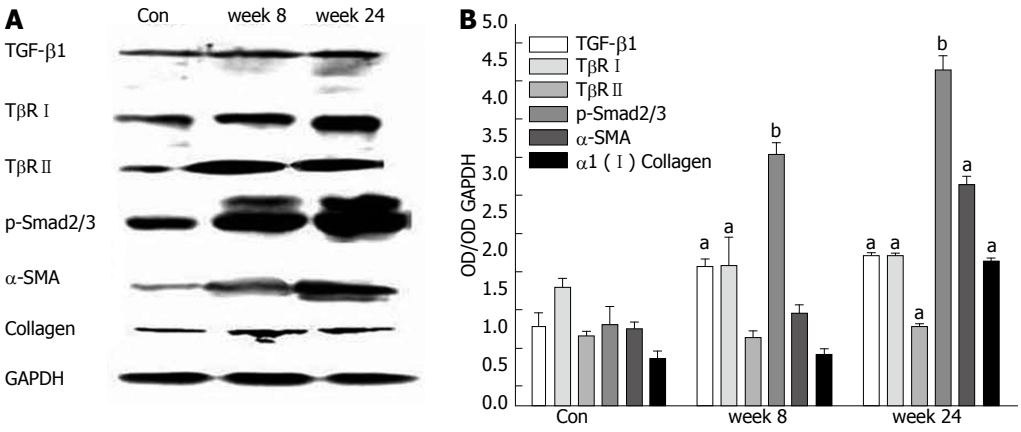

Figure 4 Immunoblot of transforming growth factor-β1, transforming growth factor-β receptor Ⅰ, transforming growth factor-β receptor Ⅱ, p-Smad2/3, α-smooth muscle actin antibody and α1 (Ⅰ) collagen. A: Western blotting analyses of transforming growth factor (TGF)-β1, TGF-β receptor Ⅰ (TβR Ⅰ), TβR Ⅱ, pSmad2/3, α-smooth muscle actin (SMA) antibody and α1 (Ⅰ) collagen expression of the liver homogenates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was an internal control for equal loading (n = 6); B: $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control mice.

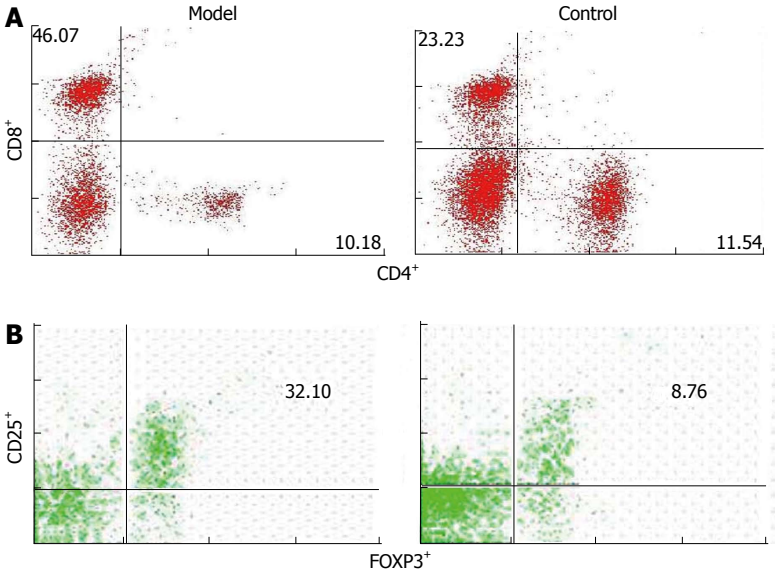

Figure 5 Lymphocytic subsets of the liver. A: The percentage of $CD4^+$ and $CD8^+$ cells in total lymphocytes population in liver; B: The percentage of $CD25^+$ $FOXP3^+$ in $CD4^+$ cells population in liver.

infiltration of $FOXP3^+$ Tregs in damaged organ or target tissues in autoimmune diseases, suggesting that suppressor cells migrate to and/or multiply at the sites of inflammation as part of immune response to combat injurious inflammation[19-21], and in liver suppress hepatic immunity to autoantigens[32]. Taken together, our study illustrates that TGF-β1 regulation of $FOXP3^+$ Tregs may be involved in the maintenance of chronic inflammation in PBC.

TGF-β1 down-regulates potentially harmful inflammatory responses in the liver, albeit at the expense of scar formation[33]. TGF-β1 signaling could induce phosphorylation of Smad2 and Smad3, which translocate into the nucleus to regulate expressions of specific target genes such as α1 (Ⅰ) collagen and α-SMA[34]. Our study demonstrated that in the liver of mouse model, the levels of TGFβ1 as well as TβR Ⅰ, TβR Ⅱ, p-Smad2/3, α-SMA and α1 (Ⅰ) collagen increased with age. These findings revealed that TGFβ1 may be involved in the fibrogenesis of the mice PBC model. Liver fibrosis occurs as a consequence of the differentiation of hepatic stellate cells (HSCs) into myofibroblasts, which is regulated by TGF β1[35]. Our study showed that the number of cells positive for α-SMA, which is a marker for myofibroblast-like cells[36], increased in aged mice in the animal model, which was coincident with increased expression of TGFβ1 and its signal molecules, supporting the finding that TGFβ1 signal pathway was involved in myofibroblast differentiation and subsequent liver fibrosis in the mouse PBC model.

In conclusion, although our data are derived from a murine model of PBC whose immunoregulation in PBC is likely to be far less complex than in human, the findings emphasize the role of TGFβ1 in development of

PBC. TGFβ1 plays a dual role in development of PBC: it suppresses inflammatory response but operates to enhance fibrogenesis. The aberrant activity of TGF-β1 signaling contributes to the development of PBC.

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COMMENTS

Background

Primary biliary cirrhosis (PBC) is an autoimmune liver disease. Recent studies suggest that transforming growth factor (TGF)-β1 signaling pathway might play an important role in the pathogenesis of PBC. However, whether TGF-β1 signaling pathway is involved in the development of PBC is still unknown.

Research frontiers

TGF-β1 plays an important role in autoimmunity and liver fibrosis, and a TGF-β1 receptor knockout mouse has been recently proposed as a model for PBC. There is strong experimental evidence that TGF-β1 is implicated in the pathogenesis of PBC, probably through deregulation of T-reg.

Innovations and breakthroughs

An animal model of PBC was developed by polyinosinic polycytidylic acids (poly Ⅰ:C) injection in genetically susceptible C57BL/6 female mice in this study. And the liver expressions of TGF-β1, TGF-β receptor Ⅰ (TβR Ⅰ), TβR Ⅱ, p-Smad2/3, monoclonal α-smooth muscle actin antibody (α-SMA) and α1 (Ⅰ) collagen in mouse model and control mice were evaluated. The relationship between TGF-β and Treg was also analyzed. The study found that TGFβ1 played a dual role in the development of PBC. The aberrant TGF-β1 signaling contributed to the development of PBC.

Applications

This study has provided new data of TGF-β1 signaling pathway involving the pathogenesis of PBC, which will pose significant impact on the understanding of the pathogeneses of PBC. Moreover, the data is the novel result of the role of TGF-β1 in the development of PBC. TGF-β1 signaling pathway is a potential target for PBC treatment.

Peer review

This paper finds that aberrant TGF-β1 signaling contributes to the development in PBC. Until now we do not have a good answer for the role of TGF-β1 signaling in PBC. These findings may be related to the immunological abnormalities of PBC while the role of TGF-β1 signaling needs further investigation.

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BRIEF ARTICLE

Consumption of gluten with gluten-degrading enzyme by celiac patients: A pilot-study

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Abstract

AIM: To assesses the safety and efficacy of *Aspergillus niger* prolyl endoprotease (AN-PEP) to mitigate the immunogenic effects of gluten in celiac patients.

METHODS: Patients with initial diagnosis of celiac disease as confirmed by positive serology with subtotal or total villous atrophy on duodenal biopsies who adhere to a strict gluten-free diet (GFD) resulting in normalised antibodies and mucosal healing classified as Marsh 0 or Ⅰ were included. In a randomised double-blind placebo-controlled pilot study, patients consumed toast (approximately 7 g/d gluten) with AN-PEP for 2 wk (safety phase). After a 2-wk washout period with adherence of the usual GFD, 14 patients were randomised to gluten intake with either AN-PEP or placebo for 2 wk (efficacy phase). Measurements at baseline included complaints, quality-of-life, serum antibodies, immunophenotyping of T-cells and duodenal mucosa immunohistology. Furthermore, serum and quality of life questionnaires were collected during and after the safety, washout and efficacy phase. Duodenal biopsies were collected after the safety phase and after the efficacy phase. A change in histological evaluation according to the modified Marsh classification was the primary endpoint.

RESULTS: In total, 16 adults were enrolled in the study. No serious adverse events occurred during the trial and no patients withdrew during the trial. The mean score for the gastrointestinal subcategory of the celiac disease quality (CDQ) was relatively high throughout the study, indicating that AN-PEP was well tolerated. In the efficacy phase, the CDQ scores of patients consuming gluten with placebo or gluten with AN-PEP did not significantly deteriorate and moreover no differences between the groups were observed. During the efficacy phase, neither the placebo nor the AN-PEP group developed significant antibody titers. The IgA-EM concentrations remained negative in both groups. Two patients were excluded from entering the efficacy phase as their mucosa showed an increase of

two Marsh steps after the safety phase, yet with undetectable serum antibodies, while 14 patients were considered histologically stable on gluten with AN-PEP. Also after the efficacy phase, no significant deterioration was observed regarding immunohistological and flow cytometric evaluation in the group consuming placebo compared to the group receiving AN-PEP. Furthermore, IgA-tTG deposit staining increased after 2 wk of gluten compared to baseline in four out of seven patients on placebo. In the seven patients receiving AN-PEP, one patient showed increased and one showed decreased IgA-tTG deposits.

CONCLUSION: AN-PEP appears to be well tolerated. However, the primary endpoint was not met due to lack of clinical deterioration upon placebo, impeding an effect of AN-PEP.

Key words: Celiac disease; Gluten; Enzyme; Prolyl endoprotease; *Aspergillus niger* prolyl endoprotease; Treatment; Adverse events; efficacy; IgA-tTG intestinal deposits

Tack GJ, van de Water JMW, Bruins MJ, Kooy-Winkelaar EMC, van Bergen J, Bonnet P, Vreugdenhil ACE, Korponay-Szabo I, Edens L, von Blomberg BME, Schreurs MWJ, Mulder CJ, Koning F. Consumption of gluten with gluten-degrading enzyme by celiac patients: A pilot-study. *World J Gastroenterol* 2013; 19(35): 5837-5847 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5837.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5837

INTRODUCTION

Celiac disease (CD) is a major health care issue affecting people of all ages, with a worldwide prevalence of approximately 1%[1]. This immune-mediated small intestinal enteropathy is triggered by gluten proteins derived from wheat, barley and rye. Celiac disease is characterised by an inflammatory immune response, resulting in small-intestinal mucosal injury and malabsorption in genetically susceptible individuals[2]. Currently, the only safe and effective treatment is a strict gluten-free diet (GFD) combined with nutritional support, which improves the health and quality of life in the vast majority of patients[3]. However, a GFD is perceived as a substantial burden, particularly due to high costs, dietary restriction, reduced social activity, and increased health worries[4].

Gluten proteins are highly abundant in proline (15%) and glutamine (35%) residues, particularly in those regions identified as immunogenic in CD[5]. The proline- and glutamine-rich peptides in gluten are relatively resistant to proteolysis by gastric, pancreatic and intestinal enzymes[6,7]. Consequently, digestion-resistant proline- and glutamine-rich peptides can reach the intestinal epithelium intact and can trigger an immune response that eventually results in mucosal damage. To eliminate such proline-rich gluten peptides, prolyl oligopeptidases, enzymes that can cleave after a proline residue in peptides, have been investigated by Shan and colleagues[6]. Such enzymes, derived from bacteria like *Flavobacterium meningosep-tum, Sphingomonas capsulate* and *Myxococcus xanthus*, were capable of breaking down toxic gluten *in vitro*[6,8,9]. These prolyl oligopeptidases are however not stable and functional under acidic conditions of the stomach[9,10] and are unlikely to degrade gluten epitopes before they reach the small intestine. Alternative enzymes that can break down gluten are derived from germinating barley and the fungus *Aspergillus niger*. From the latter a prolyl endoprotease termed *Aspergillus niger-derived* prolyl endoprotease (AN-PEP) is derived which has distinct advantages over the bacterial prolyl oligopeptidase as it degrades both whole gluten and gluten peptides into non-immunogenic residues within minutes[11,12]. Moreover, the enzyme is active between pH 2 and pH 8, with an optimum activity at pH 4-5, and is therefore effective at the pH levels present in the stomach and beyond[11,13]. Importantly, the enzyme is not degraded by pepsin in the stomach and thus remains fully functional. Mitea *et al*[12] extended these findings by showing that AN-PEP degraded toxic gluten proteins in a food matrix into non-immunogenic gluten fragments in an *in vitro* digestion model that simulates the human gastrointestinal tract. After these promising *in vitro* results, it remains to be established in CD patients whether AN-PEP can reduce the clinical response to gluten. The aim of this two-phase proof of concept study was to demonstrate the safety of AN-PEP in the first phase and the ability of ANPEP to reduce antibody and histological response to gluten consumption by CD patients in the second phase of the study. This information will be important to further develop AN-PEP as a future digestive aid for unintentional ingestion of gluten by CD patients.

MATERIALS AND METHODS

Patients

Sixteen adults with CD were recruited at the outpatient clinic of the department of Gastroenterology and Hepatology of the VU Medical Centre Amsterdam, The Netherlands. Inclusion criteria were an initial diagnosis of CD as confirmed by histological abnormalities on duodenal biopsies classified as a Marsh ⅢB or ⅢC lesion and supported by positive serology; endomysium IgA antibodies (IgA-EM) and/or tissue transglutaminase IgA antibodies (IgA-tTG). Patients were required to have well-controlled CD as evidenced by Marsh 0 or I , and normalised IgA-EM and IgA-tTG on a strict GFD for at least one year. Women at fertile age were required to take adequate contraception measures. Reasons for exclusion were: use of any anticoagulant or immunoregulatory drug within the last 6 mo; clinically suspected bleeding tendency; pregnancy or breast feeding; presence of any concurrent active infection; and IgA deficiency.

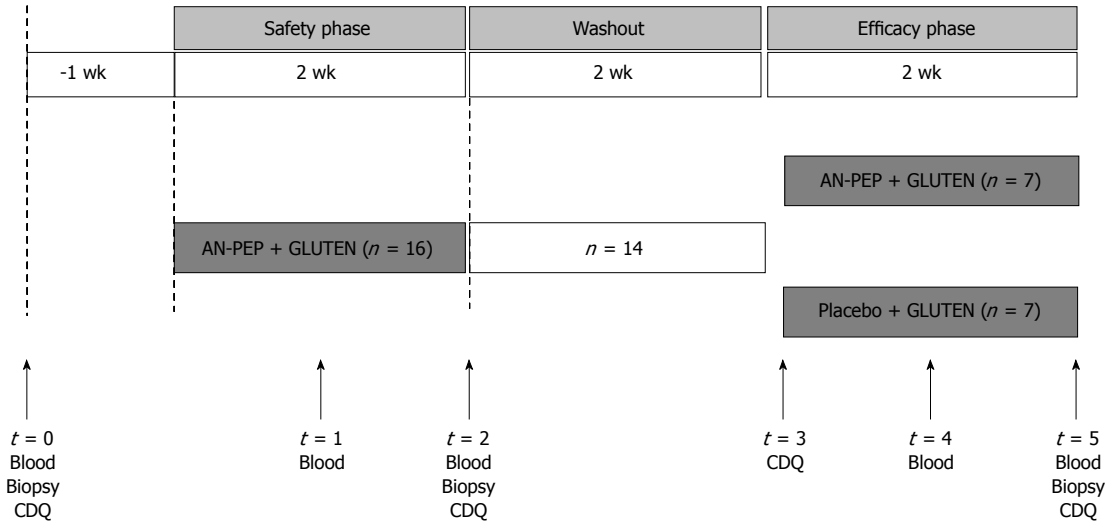

Figure 1 Study design and flowchart. In the safety phase, 16 patients daily consumed 5 pieces of toast with *Aspergillus niger* prolyl endoprotease (AN-PEP) for 2 wk while continuing their gluten-free diet (GFD). Two patients deteriorated on Marsh scores and were excluded. After a 2-wk wash-out period during which the patients continued their usual GFD, the remainder of 14 patients were randomized to the efficacy phase to receive 2 wk of toast with either AN-PEP or placebo while continuing their GFD. CDQ: Celiac disease quality.

Design and intervention

The intervention was performed between May 2008 and April 2009. The intervention consisted of two periods, each lasting 2 wk (Figure 1). The first study phase was an open-label period designed to assess the safety of high gluten intake with AN-PEP (safety phase). The second phase was a randomised, double-blind, placebo-controlled parallel-group study to assess the effect of AN-PEP on gluten-induced clinical response (efficacy phase). Sixteen patients with diagnosed CD were enrolled in the safety phase. Patients were asked to consume five pieces of toast (in total approximately 7 g gluten, Bolletje®, The Netherlands) with AN-PEP-containing topping daily in the morning for 2 wk. Patients were allowed to consume a glass of water (250 mL) with their toast. They were asked to continue their usual GFD. For ethical reasons, patients deteriorating $\geqslant$ 2 scales on the histological Marsh classification during this safety phase were not included in the efficacy phase. Between the study phases, a 2-wk washout period was introduced in which patients continued their usual GFD. Subsequently, fourteen patients were randomised in a 1:1 ratio in blocks of four in a double-blind fashion to the same amount of toast with AN-PEP-containing topping (n = 7) or placebo topping (n = 7) for 2 wk while remaining on their usual GFD. Patients' compliance with the product intake was checked by regular telephone contact.

Before and during the study phases, the patients visited the outpatient clinic five times (Figure 1). During the safety phase, blood was collected one week before (baseline), and one and two wk after start of gluten with AN-PEP consumption. During the efficacy phase, blood was collected at one and two wk after start of gluten with AN-PEP or placebo consumption. Duodenal biopsies were taken at baseline and at the end of the safety phase and the end of the efficacy phase. Both in the safety and efficacy phase, participants were asked to complete a celiac disease-specific health-related quality of life questionnaire for adults[14] at baseline and after two wk of intervention. Biopsies and blood sampled at the end of the safety phase were used as baseline values to limit the burden for the patients.

AN-PEP enzyme

The AN-PEP and placebo topping were prepared by DSM Food Specialties, Delft, The Netherlands. Both toppings (18.5 g) contained 8.2 wt% sucrose, 8.2 wt% saccharine solution (400 mg/L saccharine plus 4000 mg/L cyclamate), 0.4 wt% citric acid (Jungbunzlauer, Basel, Switzerland), 0.08 wt% potassium sorbate (Interland Chemie, Oosterhout, The Netherlands), 0.31 wt% sodium benzoate (Prolabo, Leuven, Belgium), and 1.23 wt% xanthane gum Keltrol RD (CP Kelcko, Nijmegen, The Netherlands). The AN-PEP topping contained 81.5 wt% AN-PEP enzyme concentrate corresponding with 168 Proline Protease Units of enzyme activity. The placebo topping contained 81.5 wt% distilled water with 0.06 wt% Plantex® MDA31 (colouring agent, DSM Food Specialties, Delft, The Netherlands) to match for colour differences. The aroma, flavour and consistency of the topping with AN-PEP were identical to those with placebo and both toppings could not be distinguished. Microbial counts and enzyme activity of the AN-PEP and placebo toppings were analysed monthly. All microbial counts remained below 10 CFU/g and the activity of the enzyme was maintained at 9.1 ± 0.3 PPU/g topping during 12 mo shelf life at 4 ℃. The AN-PEP and placebo toppings were identical in taste and appearance. They were pre-packed in containers (14 per box) by DSM and consecutively numbered for each patient according to the randomisation schedule (prepared by the DSM statistician).

Blinding

Each patient was assigned a random order number and received from the physician the containers in the corresponding non-transparent pre-packed box. The allocation sequence was concealed from the researcher enrolling and assessing participants in sequentially numbered sealed non-transparent envelopes. Envelopes were opened only after completion of the trial and assessments. All patients, investigators, care providers, and staff assessing outcomes were kept blind to treatment assignment.

Measurements

Mucosal biopsy immunohistology and immunophenotyping of lymphocytes, and serum antibodies were measured in the service laboratories of the VU University Medical Centre (Amsterdam, The Netherlands). Mucosal biopsy gluten-specific T-cell lines were measured in the research laboratory of the Leiden University Medical Centre (Leiden, The Netherlands). Mucosal biopsy IgA-tTG deposits were analysed at the University of Debrecen (Hungary).

Adverse event reporting: Tolerability of the gluten intake with AN-PEP or placebo was assessed by adverse event reporting to the physician during visits. All complaints were documented throughout the study. The study design did not allow for differentiation between complaints resulting from gluten or treatment. A difference in complaints between the AN-PEP and placebo group during the efficacy phase may give an indication of treatment-related effects.

Celiac-disease quality of life: All participants were asked to complete at home the CD quality of life questionnaire, which was translated into Dutch. The CD quality of life questionnaire included four disease-specific and health-related categories (emotional problems, social problems, disease-related worries, and gastrointestinal symptoms) with 7 items each[14]. Each question was weighed on a scale of 1-7 points, a high score corresponding to a high level of well-being. In total 196 points could be obtained, with a maximum of 49 points for each separate category. A change of 12 or more points on the total score or of 3 or more on the different categories was considered a clinically relevant change[14].

Mucosal biopsy immunohistology: Twelve duodenal mucosal spike biopsies were taken through upper gastrointestinal endoscopy. Four paraffin-embedded biopsies were sectioned and hematoxylin-eosin-stained for histological evaluation according to the modified Marsh classification[15]. At least two grades increase in the Marsh scale was considered a clinically significant deterioration. Six fresh biopsies were used for flow cytometric analysis and two were snap frozen in liquid nitrogen and stored.

Mucosal biopsy immunophenotyping of lymphocytes: Multiparameter flow cytometric immunophenotyping of mucosal intraepithelial and lamina propria lymphocytes was performed. These lymphocytes were isolated from six duodenal biopsy specimens per time point through chemical and enzymatic dissociation[16]. The cells were stained with fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein and allophycocyanin-labelled monoclonal antibodies directed against CD3, CD4, CD8, CD16/56, CD19, CD45, CD45RA, HLA-DR, NKG2D, CD25 and TCR gamma-delta (all from BD Biosciences, San Jose, CA, United States), and appropriate isotype controls were included. Stained cells were analysed on a 4-colour flow cytometer (FACSCalibur™, BD Biosciences) and the data were analysed using Cellquest™ software (Becton Dickinson, San Jose, CA, United States). Care was taken to analyse only viable cellular events based on light scatter properties. The mean fluorescence intensity index as compared to isotype controls was calculated for the markers included.

Mucosal biopsy gluten-specific T-cell lines: Gut-resident, gluten-reactive T-cells are a hallmark of CD. To demonstrate that all patients possessed such cells, polyclonal T-cell lines were generated from small intestinal biopsies as described[17]. The resulting T-cell lines were tested for reactivity against a pepsin/trypsin digest of gluten and a pepsin/trypsin digest of gluten that had been treated with tissue transglutaminase in a T-cell proliferation assay as described[17]. In all patients gluten reactivity could be demonstrated (not shown).

Mucosal biopsy IgA-tTG deposits: Biopsies at the end of the randomisation study phase were stained for tTG-related extracellular IgA deposits and, in case of positivity, baseline biopsies were stained as well. Twelve unfixed, 5 μm-thick frozen sections were examined per patient by double immunofluorescent labelling of IgA (green) and tTG (red) as previously described[18]. IgA is normally detected only inside plasma cells and at the luminal surface, whereas in active CD, subepithelial deposits composed of IgA-tTG are found along the surface and crypt basement membranes and around mucosal vessels, corresponding to the intestinal localisation of tTG. The CD-type IgA-tTG deposits were graded from 0 to 3 according to their intensity along the basement membranes in the villous-crypt area. As this study of the small intestinal IgA-tTG deposits is highly subjective, it was performed by an independent specialist in this field in a blind manner to greatly increase its accuracy.

Serum antibodies: Blood samples were collected by venipuncture to analyse CD-associated antibodies. Levels of IgA-tTG, gliadin IgA antibodies (IgA-AG) and gliadin IgG antibodies (IgG-AG) were determined with a standard in house enzyme-linked immunosorbent assay (ELISA), using recombinant human tissue transglutamin-

Table 1 Demographic and baseline characteristics of the safety and efficacy phase

	Safety phase	Efficacy phase	
	Gluten + AN-PEP (*n* = 16)	Gluten + placebo (*n* = 7)	Gluten + AN-PEP (*n* = 7)
Patients (*n*)	16	7	7
Gender (female:male)	12:4	5:2	6:1
Median age at inclusion, yr (range)	55 (20-68)	44 (20-68)	57 (30-64)
Median age at diagnosis, yr (range)	44.5 (0-62)	29 (0-62)	49 (26-53)
Median time on a GFD, yr (range)	7.5 (2-40)	9 (2-40)	8 (4-12)
HLA class (*n*)			
DQ2/X	12	5	5
DQ2/DQ2	2	1	1
DQ2/DQ8	1	0	1
Unknown	1	1	0
Marsh at inclusion (*n*)			
Marsh 0	10	4	3
Marsh I	6	3	4
Gastrointestinal symptoms			
Abnormal bowel sounds	4	0	2
Abdominal pain	5	3	2
Bowel distension	5	3	1
Change of defecation	4	2	6
Constipation	3	2	0
Diarrhoea	3	1	1
Dysgeusia	1	1	0
Flatulence	6	1	2
Nausea	4	2	0
Reflux	2	0	1
Vomiting	1	1	0
Weight loss	0	1	0
Total number of symptoms	38	17	15

GFD: Gluten-free diet; AN-PEP: *Aspergillus niger* prolyl endoprotease.

ase (Diarect AG, Freiburg, Germany) and gliadin extract (Sigma-Aldrich, Zwijndrecht, the Netherlands) as substrates, respectively. IgA-EM antibodies were determined by an in-house indirect immunofluorescence test according to Lerner using monkey oesophagus as substrate[19]. IgA deficiency was excluded to avoid false negative serology. In addition, in retrospect a combined test for IgA and IgG antibodies directed against human tissue transglutaminase and deamidated gliadin-derived peptides (IgA/G-DGP-tTG; tTG/DGP Screen ELISA, INOVA Diagnostics, San Diego, United States) was performed[20]. References values for antibodies were categorized into negative, dubious, weak positive, positive, and strong positive. Reference ranges for IgA-AG were < 2.4, 2.5-3.9, 4.0-20, 20-80, and > 81 U/mL, for IgG-AG, < 11, 12-20, 21-40, 41-100 U/mL, for IgA-tTG, < 2.9, 3.0-5.9, 6.0-20, 21-50, > 51 U/mL, and for IgA/G-DGP < 6.9, 7.0-10.9, 11-30, 31-100 and > 100 U/mL respectively.

Ethical approval

The study was approved by the Medical Ethics Committee of the VU Medical Centre and conducted in accordance with the guidelines of the Declaration of Helsinki. The trial has been registered in the Dutch Trial register (NTR1281) and the FDA Clinical Trial register (NCT00810654). A written informed consent was obtained from each subject before enrolment.

Statistical analysis

Data were analysed by OCS Biometric Support (Leiden, The Netherlands). Difference from baseline in mucosal immunohistology between the two groups after 2 wk as measured by Marsh classification was considered the primary outcome measure. All other parameters were considered secondary endpoints. Power analysis revealed that for the detection of a two-grade difference in the Marsh score with a power of 0.80 and a one-sided *a* level of 0.05, 14 patients were needed to finalise the study. Data were analysed in the SAS version 9.1, using both parametric and non-parametric tests depending on the nature of the data. The quality of life data were analysed with paired *t* tests to test for differences between data before and after the 1st (safety) and 3rd (efficacy) period of the study. Serological and histopathological outcome parameters were analysed with Wilcoxon signed-rank tests to determine differences between data before and after the 1st period and the Wilcoxon rank sum tests to test the treatment differences in change from baseline in the 3rd period of the study. In order to explore whether patients' baseline characteristics would predict their response to gluten (and hence to increase the chances of success in a future trial), rank correlations between baseline characteristics and outcome variables were explored in the placebo group using the Spearman Rank Correlation Coefficient (*r*) of the ranked data (analysed by DSM

statistician).

RESULTS

Baseline characteristics

The demographic and baseline characteristics of the patients are presented in Table 1. In total, 16 adults on a gluten-free diet diagnosed as having CD [median age: 55 (20-68) years] were enrolled in the study. The demographic characteristics of both treatment groups were comparable with exception of the median age at diagnosis of CD, which was 20 years higher in the AN-PEP compared to the placebo group. The median time on GFD treatment was similar in both groups. Two patients were excluded after the safety phase because of a histological deterioration of two and three Marsh grades, respectively, which for one patient returned to normal (Marsh 0) after four weeks of exclusion. The patient that did not return to normal started the study with high IgA/G-DGP-tTG values. However, other CD-related antibodies remained undetectable in these two patients. The remaining 14 patients entered and completed the efficacy phase.

When correlating the patients' baseline characteristics with their response to gluten, highly significant inverse relationships were found between the patients' time since diagnosis or time spent on a GFD and their response to gluten as measured by IgG-AG, IgA-tTG and IgA/G-DGP-tTG, and Marsh scores (data not shown).

Adverse events

No serious adverse events occurred during the trial, patients reported no severe adverse events, and no patients withdrew during the trial. Complaints that were reported during the safety and efficacy phase were of gastrointestinal nature and mostly mild and transient. The number of reported gastrointestinal complaints did not differ between the AN-PEP and placebo group (Table 1).

Celiac-disease quality of life

The mean total scores of the four categories on the CD quality of life were relatively high (145-156 out of a total score of 196) in the total group and throughout both study phases. In the safety phase, the total CD quality of life score significantly ($P = 0.04$) increased by 6 points during gluten with AN-PEP treatment. This increase was however lower than the 12-point increase that is considered a clinically relevant quality of life improvement[14]. In the efficacy phase, the individual or total CD quality of life scores of patients consuming gluten with placebo or gluten with AN-PEP did not significantly deteriorate. No differences between the groups were observed. The mean score for the gastrointestinal CD quality of life was relatively high throughout the study, indicating that gluten with AN-PEP was well tolerated.

Mucosal biopsy immunohistology

In the patients receiving gluten plus AN-PEP treatment in the safety phase, several patients showed variation in Marsh scores but overall no significant change in degree of mucosal damage, as indicated by changes in the Marsh score, was observed (Table 2). Two of 16 patients were excluded from entering the efficacy phase as their mucosa showed an increase of two Marsh steps while 14 patients were considered histologically stable on gluten with AN-PEP. Also after the efficacy phase, no significant deterioration was observed in the group consuming gluten with placebo compared to the group receiving AN-PEP.

Mucosal biopsy immunophenotyping of lymphocytes

Flow cytometric analysis of intestinal lymphocyte subsets showed no significant changes in the expression of the T-cell lineage associated markers CD3, CD4, CD8 and TCR γδ, in either the intraepithelial lymphocyte or the lamina propria lymphocyte populations of both treatment groups during the efficacy phase. The mean fluorescence index of the activation markers CD25, HLA-DR, the NK receptor and NKG2D as well as CD45RA, a marker for naïve T-cells, showed no significant change in either group.

Mucosal biopsy IgA-tTG antibody deposits

Mucosal tTG-related extracellular IgA deposits are hypothesised to be an early marker for CD activity[21]. Despite a GFD, two of seven patients started with positive staining for IgA-tTG at baseline (Table 2). Compared to baseline, IgA-tTG deposit staining increased after 2 wk of gluten intake in four out of seven patients on placebo. In the seven patients receiving AN-PEP, one patient showed increased and one showed decreased IgA-tTG deposits (Table 2, Figure 2).

Serum antibodies

Serum CD-associated antibodies (IgA-tTG, IgA-EM, IgA-AG, IgG-AG and IgA/G-DGP-tTG) were not detectable in the serum of enrolled patients at baseline (Table 2) except for one patient in which borderline levels of IgA/G-DGP-tTG were detected, which became negative after 2 wk of gluten with AN-PEP consumption. The IgA-tTG, IgG-AG, IgA/G-DGP-tTG, and IgA-EM antibody titers remained negative on gluten with AN-PEP. Three out of sixteen patients developed detectable or borderline IgA-AG levels, while 13 patients remained negative during 2-wk of gluten with AN-PEP (Table 2).

During the efficacy phase, neither the placebo nor the AN-PEP group developed significant antibody titers (Table 2). The median antibody titers after 2 wk gluten intake did not significantly differ between AN-PEP and placebo treatment. The IgA-EM concentrations remained negative in both groups.

DISCUSSION

The enzyme AN-PEP might possibly assist in digesting

Table 2 Serum antibodies, duodenal immunohistology and tTGA-A antibody deposits in the safety and efficacy phase for all patients

	Baseline						Safety phase 2 wk gluten + AN-PEP						Efficacy phase 2 wk gluten + AN-PEP or placebo						
	Serum				Biopsy		Tx	Serum				Biopsy	Tx	Serum				Biopsy	
	IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh	IgA-tTG deposits		IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh		IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh	IgA-tTG deposits
1	-	-	-	-	I	ND	A	-	-	-	-	0	P	-	-	-	-	I	0
2	-	+/-	-	-	0	0	A	-	+	-	-	I	P	-	++	+	-	IIIA	1-2
3	-	-	-	-	I	0	A	-	-	-	-	I	P	-	-	-	-	IIIA	1-2
4	+/-	-	-	+	I	1	A	-	-	-	+	0	P	+	-	-	++	I	2-3
5	-	-	-	-	0	ND	A	-	-	-	-	0	P	-	-	-	-	0	0
6	-	-	-	-	0	0	A	-	-	-	-	0	P	-	-	-	-	0	1
7	-	-	-	-	0	0	A	-	-	-	-	0	P	-	-	-	-	I	1-2
8	-	-	-	-	I	ND	A	-	-	-	-	II	A	-	-	-	-	I	0
9	-	-	-	-	0	3	A	-	-	-	-	I	A	-	+/-	-	+	I	1
10	-	-	-	-	0	ND	A	-	+/-	-	-	I	A	-	+/-	+	+	I	0
11	-	-	-	-	I	0	A	-	+	-	-	II	A	-	+/-	-	-	IIIA	1
12	-	-	-	-	I	ND	A	-	-	-	-	I	A	-	-	-	-	I	0
13	-	-	-	-	0	ND	A	-	-	-	-	0	A	-	-	-	-	0	0
14	-	-	-	-	0	ND	A	-	-	-	-	0	A	-	-	-	-	0	0
15	-	-	-	++	0	ND	A	-	-	-	++	II	E	-	-	-	++	IIIA	ND
16	-	-	-	-	0	ND	A	-	-	-	-	IIIA	E	-	-	-	-	0	ND

The serum EMA-A antibodies remained negative in all patients during the entire study. Mucosal tTGA-A deposits were graded from 0 to 3. Tx: Treatment; A: AN-PEP; P: Placebo; E: Excluded; IgA-tTG: Anti-tissue transglutaminase IgA antibodies; IgA-GA: Anti-gliadin IgA antibodies; IgG-GA: Anti-gliadin IgG antibodies; IgA/G-DGP-tTG: Anti-tissue transglutaminase and deamidated gliadin-derived peptide IgA and IgG antibodies; I-FABP: Intestinal fatty acid binding protein; ND: Not determined. -: Negative; +/-: Dubious; +: Weak positive; ++: Positive; +++: Strong positive.

unintentionally ingested amounts of gluten in those who cannot tolerate gluten. However, demonstrating a treatment effect on (small) clinical deterioration induced by small amounts of gluten in the placebo group may be difficult. Therefore, in this proof of principle study, the enzyme was given to patients consuming large amounts of gluten in a relative small period of time. A two-week safety phase (AN-PEP + gluten) preceded the randomization for AN-PEP or placebo as requested by the medical ethical commission due to concerns about such a high dose of gluten consumption. Unfortunately, the primary aim of the study was not met as the placebo arm did not show any deterioration after 2 wk of gluten consumption. With hindsight, the study should possibly have been designed for a much longer period of time with many more patients.

The baseline characteristics were balanced between groups except for median age at diagnosis, which was 20 years higher in the AN-PEP compared to the placebo group. However, this is unlikely to have influenced the study outcome as no relationship between the age of diagnosis and the response to gluten was observed (data not shown).

The safety phase showed that AN-PEP treatment, when consumed with a high dose of about 7 g of gluten for 2 wk, was safe in patients and no severe adverse events were reported. The CD quality of life scores remained relatively high during 2 wk consumption of gluten and AN-PEP indicating that patients' general well-being remained high. Serum antibodies of the sixteen patients did not increase when consuming AN-PEP with 7 g of gluten for 2 wk. Also, histology of the biopsies of the majority of patients (fourteen) showed no deterioration while two patients developed increased Marsh scores, however not accompanied by increased antibodies. The safety phase was subject to a so-called "ceiling effect" because patients entered the study on a GFD reflecting relatively healthy baseline values, limiting the ability to demonstrate any further improvement by AN-PEP.

Patients in the placebo group did not show significant deterioration on any of the measured clinical variables after a 2-wk gluten challenge, indicating that 2 wk of gluten challenge is insufficient to induce a clear clinical response in this population of celiac patients. Due to lack of response to gluten in the placebo arm, no treatment effect of

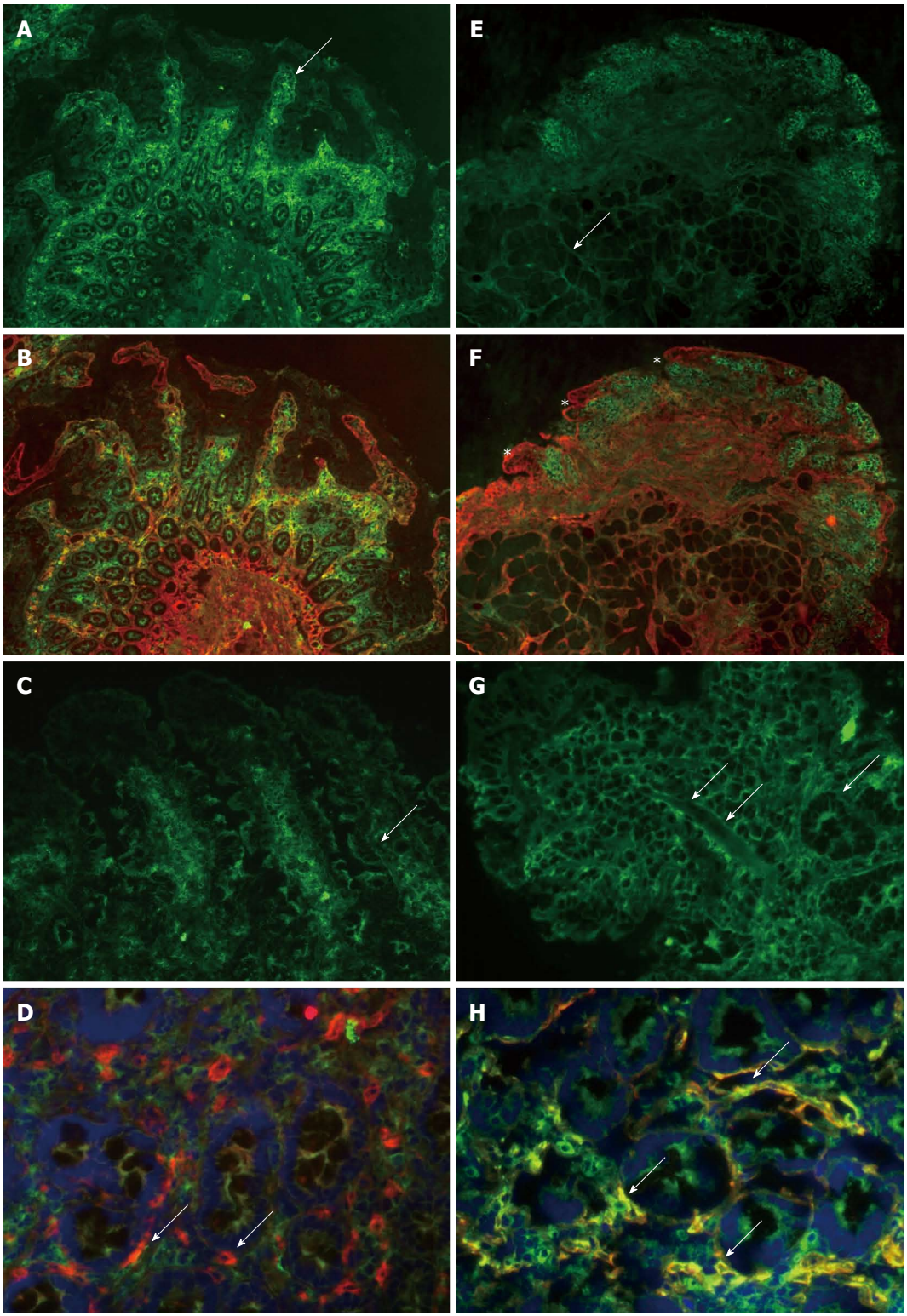

Figure 2 Small intestinal tissue transglutaminase IgA antibody deposits (rated on a scale 0-3) in two patients at baseline and after randomization to *Aspergillus niger* prolyl endoprotease and placebo respectively. A: Baseline evaluation of patient 1 showed preserved villous architecture (arrow), with intense, grade 3 IgA depositions (green) subepithelially and around crypts; B: This deposition merges to yellow indicating co-localisation with tTG shown in red; C: In this patient, IgA deposits diminished after 2 wk *Aspergillus niger* prolyl endoprotease (AN-PEP) treatment to grade 1, when only faint and patchy antibody deposition was seen (arrow); D: tTG appeared in red in this AN-PEP-treated patient (arrow) in the absence of IgA deposition. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue); E: Baseline evaluation of patient 2 showed preserved villous architecture with faint, grade 1 IgA deposition in the deep mucosal layer around Brunner glands (arrow); F: This deposition were not sufficient to obtain a yellow colour at merging with tTG shown in red (asterisk); G: In this patient, IgA deposition increased to grade 2 subepithelially (arrow) after 2 wk placebo; H: IgA deposition increased to grade 3 in the crypt region after 2-wk placebo (arrow). IgA deposition co-localised with tTG to intense yellow (arrow). The cell nuclei were stained with DAPI (blue).

AN-PEP could be detected. The measured serum levels of IgA-tTG, IgA-EM, and IgA/G-DGP-tTG antibodies are considered sensitive markers of CD and should be able to detect subtle immunogenic effects of gluten. Similarly, the CD-specific quality of life questionnaire is considered a CD-specific measure of quality of life and should also be able to pick up relevant changes in health[14]. However, histological examination of small intestinal biopsies may be less reliable than CD-associated antibodies due to heterogeneous distribution of lesions, low grade histopathology, and intra- and interobserver variability[22]. Interestingly, measures of clinical response to gluten (Marsh scores, antibody titres, quality of life scores) did not correlate in this study, which may in part be explained by the lack of response to gluten. The IgA antibody reactivity to small intestinal mucosa tTG has been considered to be an early marker for gluten-induced pathology in CD patients[23]. It was observed that intestinal IgA-tTG deposits can be detected in latent CD patients in whom the mucosal villous architecture is still intact, and that the intensity of these mucosal deposits decreased after adherence to a GFD and increased after gluten consumption. Although numbers were low, mucosal IgA-tTG deposits increased in four patients on placebo and one on AN-PEP and decreased in one patient on AN-PEP, compared to baseline values, suggesting that AN-PEP may mitigate gluten exposure.

Some gastrointestinal-related symptoms, mostly mild and transient, were reported during gluten challenge and symptoms between the two groups were comparable suggesting no treatment-related effects on gastrointestinal symptoms. Besides the substantial gluten intake, emotional stress as a consequence of having to ingest gluten might have triggered some of the reported gastrointestinal complaints.

The celiac patients consumed approximately 7 g of gluten daily, which is about half of the average adult daily gluten intake in The Netherlands[24]. Despite this high gluten dose, no substantial histological, serological, or symptom changes were observed with placebo after 2 wk. In another study[25] in which adult CD patients consumed approximately 3.5 g/d of gluten from cracker biscuits for 2 wk, only few patients consuming gluten on placebo showed deterioration on histology, serology, and symptoms. Two other studies investigating a gluten challenge in adult patients, based on either lower gluten intake (2.5-5.0 g/d for at least 3 mo)[26] or comparable gluten intake (4 slices of white bread daily; approximately 8 g/d)[27] showed that a moderate gluten intake can be tolerated by some patients for several weeks-to-months without significant changes in symptoms[27], serology[26] and histology[26,27]. The time to serological and mucosal relapse and recovery after gluten re-introduction and elimination, respectively, can be highly variable among adult CD patients from several weeks up to many years[27-30]. Excluding 2 out of 16 patients that may have been more sensitive to gluten from the efficacy phase may, to a small extent, have caused sample bias by selecting patients being less sensitive to gluten. Nevertheless, the same population of patients that entered the efficacy phase was randomly allocated to the AN-PEP or placebo arm. Also attrition bias can be excluded since all patients remained in the study. The lack of substantial clinical response to gluten observed in this study indicates that a longer gluten challenge is likely necessary to induce a significant clinical response to gluten in the majority of patients. For the same reason a longer wash-out period should be considered. Moreover, unresponsiveness to gluten of patients being diagnosed for more than 10 years ago, suggests that future studies may benefit from selecting more recently diagnosed patients.

In conclusion, AN-PEP appeared to be safe in celiac patients. More patients and gluten challenge for a longer period of time seem to be required to induce significant clinical changes and to confirm whether the tendency of AN-PEP to reduce small bowel IgA-tTG deposits is of clinical significance. These results together with previous *in vitro* evidence that AN-PEP efficiently degrades gluten under simulated gastrointestinal conditions warrant confirmation in a larger trial.

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COMMENTS

Background

The only currently available treatment for celiac disease consists of life-long dietary exclusion of gluten, perceived as a substantial burden particularly due to high costs, dietary restriction, reduced social activity, and increased health worries. Alternative treatment modalities that reduce the need of dieting focus on modification of dietary components, enzymatic degradation of gluten, inhibition of intestinal permeability and modulation of the immune response. Following this, a previous report showed that the gluten-degrading *Aspergillus niger-derived* prolyl endoprotease (AN-PEP) degraded toxic gluten proteins in a food matrix into non-immunogenic gluten fragments in an *in vitro* digestion model that simulates the human gastrointestinal tract.

Research frontiers

This is the first randomised double-blind placebo-controlled pilot-study evaluating the safety and efficacy of AN-PEP in celiac disease.

Innovations and breakthroughs

The celiac disease quality of life scores remained relatively high during 2 wk consumption of gluten and AN-PEP indicating that patients' general well-being remained high. The enzyme AN-PEP might possibly assist in digesting unintentionally ingested amounts of gluten in those who cannot tolerate gluten. However, the primary aim of the study was not met as the placebo arm did not show any deterioration (small intestinal mucosa, celiac disease associated antibodies, and quality of life) after 2 wk of gluten consumption. Although numbers were low, mucosal IgA-tTG deposits increased in 4 patients on placebo and one on AN-PEP and decreased in one patient on AN-PEP, compared to baseline values, suggesting that AN-PEP may mitigate gluten exposure.

Applications

The lack of substantial clinical response to gluten observed in this study indicates that more patients and gluten challenge for a longer period of time seem to be required to induce significant clinical changes and to confirm whether the tendency of AN-PEP to reduce small bowel IgA-tTG deposits is of clinical significance. These results together with previous *in vitro* evidence that AN-PEP efficiently degrades gluten under simulated gastrointestinal conditions warrant

confirmation in a larger trial.

Peer review

The aim of the present study was to examine the safety and efficacy of AN-PEP to reduce the clinical response to gluten in patients with coeliac disease. The safety study showed that AN-PEP was safe and well tolerated by patients with celiac disease. Data on time to serological and mucosal relapse and recovery after gluten re-introduction and elimination show highly variable results, varying from several weeks up to many years. Even though the primary endpoint was not met, this study is of interest and warrant support for such an approach.

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BRIEF ARTICLE

Laparoscopic management of totally intra-thoracic stomach with chronic volvulus

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Abstract

AIM: To evaluate the outcomes of patients who underwent laparoscopic repair of intra-thoracic gastric volvulus (IGV) and to assess the preoperative work-up.

METHODS: A retrospective review of a prospectively collected database of patient medical records identified 14 patients who underwent a laparoscopic repair of IGV. The procedure included reduction of the stomach into the abdomen, total sac excision, reinforced hiatoplasty with mesh and construction of a partial fundoplication. All perioperative data, operative details and complications were recorded. All patients had at least 6 mo of follow-up.

RESULTS: There were 4 male and 10 female patients. The mean age and the mean body mass index were 66 years and 28.7 kg/m^2, respectively. All patients presented with epigastric discomfort and early satiety. There was no mortality, and none of the cases were converted to an open procedure. The mean operative time was 235 min, and the mean length of hospitalization was 2 d. There were no intraoperative complications. Four minor complications occurred in 3 patients including pleural effusion, subcutaneous emphysema, dysphagia and delayed gastric emptying. All minor complications resolved spontaneously without any intervention. During the mean follow-up of 29 mo, one patient had a radiological wrap herniation without volvulus. She remains symptom free with daily medication.

CONCLUSION: The laparoscopic management of IGV is a safe but technically demanding procedure. The best outcomes can be achieved in centers with extensive experience in minimally invasive esophageal surgery.

Key words: Laparoscopic procedure; Hiatal hernia; Stomach; Volvulus; Mesh repair

Core tip: Migration of the whole stomach in to the chest cavity by rotating its longitudinal or transverse axis, namely "intra-thoracic gastric volvulus", is a very rare type of giant hiatal hernias and is associated with catastrophic complications. Laparoscopic repair of this rare condition is the most technically demanding procedure among the benign foregut surgeries. With careful attention the details, such as total excision of the hernia sac, provision of an adequate esophageal length with full mobilization of the esophagus, tensionless hiatoplasty, and a floppy fundoplication, long-term success is possible

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INTRODUCTION

It is difficult to identify the true incidence of hiatal hernia

because of the absence of symptoms in a large number of patients. Hiatal hernia is most commonly associated with gastro-esophageal reflux disease (GERD), and GERD affects millions of people worldwide[1]. Ninety-five percent of hiatal hernias are the small sliding type (type Ⅰ) and are not associated with life threatening complications. The remaining 5% are classified as paraesophageal and mixed types (types Ⅱ and Ⅲ, respectively) both of which are known as giant hiatal hernias.

Landmark articles on giant hernias were published by Skinner *et al*[2] in 1962 and Hill[3] in 1973. They reported mortality rates exceeding 27% due to catastrophic complications of paraesophageal hernia (PEH) such as obstruction, strangulation, perforation and bleeding. Although still controversial, many surgeons recommend elective surgical repair even in elderly asymptomatic patients with PEH[4].

After Cuschieri *et al*[5] performed the first laparoscopic PEH repair, many surgeons reported successful results with less than 1% mortality[6,7]. All studies have shown that laparoscopic repair of giant hiatal hernias is a safe but technically demanding procedure. Because of the rarity of this disease and the lack of randomized trials comparing different surgical approaches, controversy exists regarding which surgical approach should be preferred. Choices regarding the type of surgical procedure include trans-abdominal *vs* trans-thoracic, open *vs* laparoscopic, hiatal closure with primary suture *vs* the use of meshes and whether fundoplication is necessary[8,9].

Many previous publications addressed the management of PEH, but there is a distinct subgroup of patients who represent the end stage of all types, which occurs when the whole stomach migrates into the thorax by rotating 180 degrees around its longitudinal or transverse axis, namely "intra-thoracic gastric volvulus (IGV)". Surgical repair of this rare disorder is most likely the most technically difficult procedure among the benign foregut diseases, even for experienced foregut surgeons. The present article focuses on this subgroup of patients who have IGV.

MATERIALS AND METHODS

Patient selection

The study was conducted at our anti-reflux therapy center, which is a specialized tertiary referral center for the diagnosis and treatment of GERD. A retrospective review of a prospectively collected database of patient medical records identified 14 patients who underwent laparoscopic repair for a totally intra-thoracic stomach with chronic volvulus. IGV was defined as transmigration of the whole stomach into the thorax by a 180 degree around its longitudinal or transverse axis (Figure 1). Surgical consent was obtained from all patients after detailed information was given by a senior surgeon. The preoperative evaluation included an upper gastrointestinal endoscopy, thoraco-abdominal computed tomography (CT) and a barium esophagram. We do not perform a routine 24-h pH study or manometry as the results do not change our treatment strategy in IGV cases. Pulmonary function testing was performed in patients with pulmonary symptoms, such as shortness of breath, to determine whether breathing problems were due to restriction of the lung by an intra-thoracic stomach or to intrinsic lung disease.

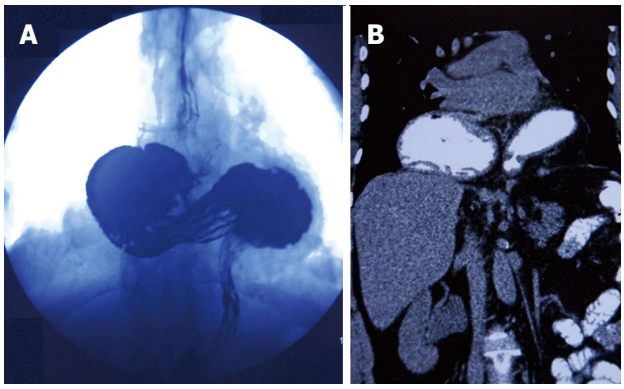

Figure 1 Computed tomography. A: Preoperative barium swallow study shows the transmigration of the entire stomach; B: Coronal view of intra-thoracic gastric volvulus.

Surgical technique

All patients were admitted on the day of the surgery and underwent a laparoscopic hiatal hernia repair procedure after an overnight fast. Patients received prophylaxis by subcutaneous low-molecular-weight heparin administered routinely during the induction of anesthesia in addition to compression stockings.

Patients were placed in the modified lithotomy position with the surgeon between the legs and the assistant on the left side. The first 10-mm optic port was placed using the open Hasson technique in the upper midline approximately one third of the way from the umbilicus to xiphoid. An additional three 10-mm and two 5-mm trocars were placed in the upper abdomen after a pneumoperitoneum was established to a pressure of 13-15 mmHg. Unlike patients who undergo an antireflux surgery, trocar placement was higher in the abdomen and was very similar to the placement in obese GERD patients we previously reported[10].

Following liver retraction, the hiatal hernia was examined. Then the operating room table was placed in reverse Trendelenburg to allow an easier reduction. The herniated stomach was reduced into the abdomen as much as possible with atraumatic graspers in a hand-over-hand fashion. The dissection was started by dividing the gastro-hepatic ligament and exposing of the right crus. If a dominant left hepatic artery larger than 2 mm was seen, the dissection was started just above the vessel. There was no attempt to dissect the hernia sac or to find the esophagus at this stage. The hernia sac was identified at the junction of the left crus and stomach. Finding a fine areolar plane between the sac and surrounding mediastinal tissues was a landmark following the division of the hernia sac. Care was taken to identify the vagal nerves

Table 1 Patients demographics and perioperative findings

Age, yr (range)	66.7 ± 4.7 (61-76)
Gender	
Male	4 (28.6)
Female	10 (71.4)
BMI (kg/m^2)	28.7 ± 7.6
Symptoms	
Early satiety	14 (100)
Postprandial discomfort	14 (100)
Shortness of breath	2 (14.2)
Heartburn	2 (14.2)
Duration of operation (min)	234.9 ± 59.2
Day of discharge (d)	2.6 ± 1.4
Minor complication	4 (28.6)
Recurrence	1 (7.1)
Mean follow-up (mo)	29.4 ± 19

Data are expressed as absolute numbers (percentage) or mean ± SD. BMI: Body mass index.

and pleura to avoid any injury during the dissection of the mediastinum. Full division and removal of the sac was performed in all patients.

Following the sac removal, a circumferential dissection of the esophagus to the level of the inferior pulmonary veins was performed to achieve adequate intra-abdominal esophagus length. The final assessment of the esophageal length was conducted after careful dissection of the fat pad of the gastroesophageal junction (GEJ) with the operating table in a level position and with a 6-8 mmHg pneumoperitoneum. Collis gastroplasty (CP) was not routinely performed prior to fundoplication.

Once the esophageal dissection was completed, a crural repair with silk sutures was performed. Care was taken to avoid any tension over the crura during the repair, and we aimed to secure the integrity of the muscle. Reinforced hiatoplasty with prosthetic grafts was routinely performed. A U-shaped monofilament polypropylene graft (Prolene; Ethicon, Ltd.) was used for the reinforced hiatoplasty. The mesh was fixed to the diaphragm by a laparoscopic tacker. A fundoplication was performed during the procedure to avoid postoperative acid reflux. A partial posterior fundoplication, namely "Toupet fundoplication", was the procedure of choice. We believe a better fixation of the gastric fundus is achieved with more sutures to the esophagus and crura using the Toupet procedure. Our partial fundoplication technique was standardized and reported elsewhere[11]. Briefly, the right side of the wrap was fixed to the esophagus using two silk sutures. The left part of the wrap was sutured to the anterior side of the esophagus by two or three sutures, and a single suture was used to fix the upper side of the wrap to the upper edge of the hiatus. There was no attempt to divide the short gastric vessels as the gastric fundus is too mobile due to long-term herniation.

Postoperative period

The length of postoperative observation in the intensive care unit depended on patient co-morbidities and the operation length. All patients were discharged on the second postoperative day unless problems occurred. Patients received liquids on the first postoperative day, after an esophagram was obtained. All patients were seen at intervals of 1 wk and 2 mo after surgery and yearly thereafter. A barium esophagram was performed during annual follow-up evaluations.

RESULTS

Fourteen consecutive patients underwent a laparoscopic repair of IGV. There was no mortality, and none of the cases were converted to an open procedure. The duration of follow-up was 29.4 mo. There were 4 male and 10 female patients. The mean age and mean body mass index were 66.7 years and 28.7 kg/m^2, respectively. All patients presented with epigastric discomfort and early satiety. Only 2 patients had additional reflux symptoms of heartburn and 2 had shortness of breath. The demographic characteristics of the patients are outlined in the Table 1.

The mean operative time was 234.9 min, and the mean length of hospitalization was 2.6 d. There were no intraoperative complications. Four minor complications occurred in three patients. One patient had pleural effusion and subcutaneous emphysema that spontaneously resolved within 2 wk. One patient had postoperative dysphagia that resolved within 6 wk without any intervention. One patient had postoperative delayed gastric emptying that began in the first postoperative week. She was treated with medical therapy and her complaints resolved within 6 mo. One patient presented with recurrent heartburn 6 mo postoperatively, and a wrap herniation was diagnosed with gastroscopy and barium swallow studies. She remains symptom free with daily proton pump inhibitor usage.

DISCUSSION

An IGV is an uncommon entity and it occurs when the entire stomach migrates into the thorax through a giant hiatal defect by rotating around its longitudinal or transverse axis. Whether this rare condition is an extension of a PEH or an evolution of a longstanding sliding hernia is subject to controversy and is beyond the scope of this article. IGV is the end stage of all hiatal hernia types before catastrophic complications occur.

The clinical features of giant hiatal hernias are nonspecific and the majority of patients are asymptomatic. Dysphagia, heartburn, postprandial discomfort and chest pain are the most common presenting symptoms[12]. Patients presenting with chest pain usually undergo a cardiac work-up and a PEH is incidentally found in chest scans. Patients with IGV are usually symptomatic, and in our study all patients presented with early satiety and postprandial discomfort.

We usually start with a gastroscopy in the preoperative work-up. In addition to detecting esophagitis and/or Barrett metaplasia, an upper gastrointestinal endoscopy

Table 2 Laparoscopic repair of intra-thoracic gastric volvulus: Literature review

Ref.	*n*	Presentation	Follow-up	Mesh	Fundoplication procedure	Outcome
Inaba *et al*[15]	1	Upper abdominal pain	4 yr	PTFE	Toupet	Cure
Gökcel *et al*[16]	7	-	5 mo	PTFE	Anterior semi fundoplication	One recurrence without volvulus
Salameh *et al*[17]	1	Chest discomfort, inability to belch	1 yr	None	Nissen	Cure
Malik *et al*[18]	2	Epigastric pain, vomiting, bloating	1 yr	None	Nissen	Cure (PEG tube was placed in one patient and removed after 6 mo)
Rathore *et al*[19]	1	Chest pain, shortness of breath	1 yr	None	None	Cure
Golash[20]	1	Epigastric pain, inability to eat	6 mo	Polypropylene	Nissen+ anterior gastropexy	Cure
Iannelli *et al*[21]	1	Epigastric pain, vomiting	18 mo	-	Nissen	Cure
Krahanbuhl *et al*[22]	3	Epigastric pain, vomiting	21 mo	None	Nissen + anterior gastropexy	One recurrence with volvulus
Katkhoudan *et al*[23]	8	Epigastric pain, early satiety	16 mo	None	Nissen	One recurrence without volvulus

PEG: Percutaneous endoscopic gastrostomy.

can reveal other concomitant gastric neoplasias as the majority of patients are over 65 years old with non-specific symptoms. Unfortunately, total gastroscopy cannot be performed in most patients with IGV even under general anesthesia. Following gastroscopy, we obtain a radiographic evaluation with a barium swallow study and thoraco-abdominal CT. We think the barium swallow is very useful in identifying the presence and the type of volvulus, the location of the GEJ and in assessing the length of the esophagus. CT imaging is useful to determine possible associated organ herniation and to rule out of diaphragmatic hernia. Preoperative evaluation of patients with a pH meter and manometry is controversial. Fuller and co-workers reported 60% of patients with a giant hiatal hernia had pathological acid reflux despite the absence of typical symptoms[13]. Schieman *et al*[14] recommend routine pH meter and manometry to reveal possible concomitant reflux. We do not perform routine pH meter or manometry in our clinical practice as the results do not change our treatment strategy.

In 2013, there remains no consensus among foregut surgeons regarding the optimal surgical approach to giant hiatal hernias. The approaches include trans-abdominal *vs* trans-thoracic procedures, open *vs* laparoscopic procedures, hiatal closure with primary suture *vs* the use of meshes, fundoplication, gastroplasty and total sac excision. Because of the rarity of this disease only small series and case reports exist in the literature (Table 2). As we had extensive experience in more than 1000 anti-reflux operations, a laparoscopic approach was the procedure of choice in our series. Some surgeons advocate the transthoracic approach especially in emergency cases[24]. The improved ability to separate adhesions between the hernia sac and pleura is the main advantage of trans-thoracic repair. In recent years, successful thoracoscopic repair of intrathoracic stomach has started to appear in the literature[25]. The surgeon's experience seems to be the most important consideration in choosing the procedure.

The debate over total excision of the hernia sac is the least controversial issue. Many surgeons believe total excision of the sac eliminates the tension on the GEJ and minimizes the risk of recurrence. Edye *et al*[26] addressed this issue and reported 20% early period recurrence in patients without sac excision. Although the total excision of the sac decreases the recurrence rates, some surgeons prefer to leave the distal part of the sac as a fail-safe measure to counter difficulties in dissecting nearby pleura and vagal nerves. We believe total excision is the critical step of the operation in patients with IGV, as reducing the volvulus can only be achieved by total excision. It may be very difficult when the vagus is partly adherent to the sac, especially anteriorly, and one of our patients had postoperative delayed gastric emptying after a demanding dissection. Her complaints spontaneously resolved after 6 mo, and we believe vagal injuries may not result in long-term clinical sequelae.

Short esophagus was first described in 1957[27], and since then its pathophysiology, importance and management have remained a subject of clinical debate. Hypothetically, the inflammation of the posterior mediastinum due to the intra-thoracic stomach results in adhesion that causes esophageal shortening. The associated acid reflux can lead to chronic inflammation and fibrosis in the connective tissues that finally results in esophageal shortening. Despite the various attempts, specific criteria allowing surgeons to preoperatively identify short esophagus and to determine which patients will need a CP do not exist[28,29]. Although CP has become a more commonly used procedure in the past decade[30], some surgeons believe that there is no need to perform CP with an adequate esophageal dissection[31]. If a 2.5-3 cm intra-abdominal esophagus can be achieved by mediastinal dissection, there is no need to perform a Collis procedure. There is a tendency to overestimate the esophageal length during a laparoscopy. The pneumoperitoneum elevates the diaphragm and misleads surgeons. Surgeons should keep in mind that these maneuvers can lead to an overestimate of intra-abdominal esophageal length. A CP was not needed in our experience. In one patient, we suspected an esophageal shortening based on a preoperative up-

per intestinal series. After mobilization of the esophagus and careful dissection of the fat pad over the GEJ, we thought we had achieved an adequate esophageal length. Unfortunately, she was the patient who presented with recurrence.

The use of prosthetic grafts for a reinforced hiatoplasty is another controversial issue in the treatment of giant hiatal hernias. The main point of controversy includes what shape, size and type of mesh should be used, and whether it should be used routinely, or in selected cases. Shamiyeh and co-workers addressed this issue by calculating the mean hiatal surface area (HSA)[32]. The authors found the average HSA was 5.84 cm^2 and suggested HSA can be used for the decision to use mesh. Although the use of a prosthetic mesh seems to significantly reduce the risk for recurrence[33,34], it is not free of complications. Erosion into the gastrointestinal organs is the most feared complication when a mesh is used in the hiatus. Until recently, only a few mesh erosions were reported as single cases in the last 15 years[35]. In 2009, Stadlhuber *et al*[36] reported 28 patients with mesh complications by gathering case data from the expert esophageal surgeons. The authors suggested that the incidence mesh complications may be greater than estimated. Reinforced hiatoplasty has become routine in our early experience, even in GERD patients with small hiatal hernias. U-shaped polypropylene grafts were the preferred type of mesh. We did not observe a mesh complication in more than 700 patients. Because of the fear of mesh erosion, we used grafts more selectively after we read Stadlhuber's paper.

Adding a fundoplication procedure after the repair of the hiatus is also an issue of debate. Some surgeons recommend its selective application in patients with associated GERD[37]. Others advocate routine application because extensive dissection of the esophagus will result in GERD[38]. Nissen fundoplication is the most commonly used procedure. We routinely performed Toupet fundoplication in the present series. We can provide more fixation of the gastric fundus with more sutures. As the majority of these patients are over 65 years old, they have baseline esophageal dysmotility, and total fundoplication may result in dysphagia[39].

As a result of negative intrathoracic pressure, there is always a tendency for the wrap to migrate back to the thorax following the repair of giant hiatal hernias. Anterior gastropexy was recommended to overcome this problem. Ponsky *et al*[40] reported a prospective study of 31 patients who underwent laparoscopic PEH repair. The authors did not observe recurrence during the 21 mo follow-up period. We believe gastropexy should not be an option in patients who have IGV, as it may create a new axis that can lead to intra-abdominal volvulus.

In conclusion, laparoscopic management of IGV is a safe procedure and should be the first option in the treatment algorithm. With careful attention the details, such as total excision of the hernia sac, provision of an adequate esophageal length with full mobilization of the esophagus, tensionless hiatoplasty, and a floppy fundoplication, long-term success is possible. This procedure is most likely the most technically demanding procedure among the benign foregut diseases and requires advanced laparoscopic skills. The best outcomes can be achieved by surgeons with extensive experience, especially in laparoscopic anti-reflux surgery, as there is no learning curve for this rare condition.

COMMENTS

Background

Giant hiatal hernias are frequently associated with catastrophic complications such as obstruction, perforation and bleeding. Intra-thoracic gastric volvulus (IGV) is the rarest type and represents end stage of giant hiatal hernias before these complications occur.

Research frontiers

Minimally invasive approaches for the treatment of foregut diseases are increasing worldwide. Laparoscopic management of IGV is probably most technically demanding procedure among the benign foregut diseases. The authors have focused on technically details and preoperative work-up in the management of this uncommon condition.

Innovations and breakthroughs

Because of the rarity of IGV there is still no prospective randomize study which compares different surgical approaches and controversy exists regarding which surgical approach should be preferred such as; trans-abdominal *vs* transthoracic, open *vs* laparoscopic, hiatal closure with primary suture *vs* the use of meshes and the necessity of fundoplication. Laparoscopic approach was the procedure of choice as the authors have extensive experience in laparoscopic anti-reflux surgery. Total sac excision, tensionless hiatoplasty with mesh and Toupet fundoplication were performed in all patients without mortality and minimal morbidity.

Applications

With careful attention the details, laparoscopic management of IGV is a safe procedure.

Terminology

IGV is defined as transmigration of the whole stomach into the thorax by rotating 180 degrees around its longitudinal or transverse axis.

Peer review

The authors have described their experience well in the management of this rare type of giant hiatal hernia.

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BRIEF ARTICLE

Role of *Salmonella enterica* exposure in Chilean Crohn's disease patients

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Abstract

AIM: To study the association between exposure to *Salmonella enterica* (SE) and Crohn's disease (CD) and its clinical implications in Chilean patients.

METHODS: Ninety-four unrelated Chilean CD patients from CAREI (Active Cohort Registry of Inflammatory Bowel Disease) presenting to a single inflammatory bowel disease (IBD) unit of a University Hospital were prospectively included in this study. A complete clinical evaluation, including smoking history, was performed at the initial visit, and all the important data of clinical evolution of CD were obtained. Blood samples from these CD patients and 88 healthy sex- and age-matched control subjects were analyzed for exposure to SE and for their *NOD2*/*CARD15* gene status using the presence of anti-*Salmonella* lipopolysaccharide antibodies [immunoglobulin-G type (IgG)] and polymerase chain reaction (PCR), respectively. We also evaluated exposure to SE in 90 sex- and age-matched patients without CD, but with known smoking status (30 smokers, 30 non-smokers, and 30 former smokers).

RESULTS: CD patients comprised 54 females and 40 males, aged 35.5 ± 15.2 years at diagnosis with a mean follow-up of 9.0 ± 6.8 years. CD was inflammatory in 59 patients (62.7%), stricturing in 24 (25.5%) and penetrating in 15 (15.5%). Thirty cases (31.9%) had lesions in the ileum, 29 (30.8%) had ileocolonic lesions, 32 (34.0%) had colonic lesions and 23 (24.4%) had perianal disease. Sixteen CD patients (17%) were exposed to SE compared to 15 (17%) of 88 healthy control subjects (P = 0.8). Thirty-one CD patients (32.9%) were smokers, and 7 (7.4%) were former smokers at diagnosis. In the group exposed to SE, 10 of 16 patients (62.5%) were active smokers compared to 21 of 78 patients (26.9%) in the unexposed group (P = 0.01). On the other hand, 10 of 31 smoking patients (32%) were exposed to SE compared to 5 of 56 nonsmoking patients (9%), and one of the seven former smokers (14%) (P = 0.01). In the group of 90 patients without CD, but whose smoking status was known, there was no differ-

ence in exposure to SE [3 of 30 smokers (10%), 5 of 30 non-smokers (16%), and 5 of 30 former smokers (16%); *P* = 0.6]. There were no differences in disease severity between CD patients with and those without anti-SE IgG antibodies, estimated as the appearance of stricturing [2 (12.5%) *vs* 22 (28.2%); *P* = 0.2] or penetrating lesions [2 (12.5%) *vs* 13 (16.6%); *P* = 1.0]; or the need for immunosuppressants [11 (68.7%) *vs* 55 (70.5%); *P* = 1.0], anti-tumor necrosis factor therapy [1 (6.2%) *vs* 7 (8.9%); *P* = 1.0], hospitalization [13 (81.2%) *vs* 58 (74.3%); *P* = 0.7], or surgery [3 (18.7%) *vs* 12 (15.3%); *P* = 0.3), respectively]. No other factors were associated with SE, including *NOD2/CARD15* gene status. Seventeen CD patients (18%) had at least one mutation of the *NOD2/CARD15* gene.

CONCLUSION: Our study found no association between exposure to SE and CD. We observed a positive correlation between SE exposure and cigarette smoking in Chilean patients with CD, but not with disease severity.

Key words: Crohn's disease; *Salmonella*; Infection; Tobacco; Smoking; Environmental factors

Core tip: The role and clinical implications of *Salmonella enterica* (SE) in Crohn's disease (CD) are controversial and currently unknown. We evaluated the role of exposure to SE in a cohort of Chilean patients suffering from CD. Although our study showed no association between SE exposure and CD, we observed a positive correlation between SE exposure and cigarette smoking in CD patients, but not with disease severity. These data provide evidence that more precisely defines the real role of *Salmonella* infection, an important environmental factor in CD.

Alvarez-Lobos M, Pizarro DP, Palavecino CE, Espinoza A, Sebastián VP, Alvarado JC, Ibañez P, Quintana C, Díaz O, Kalergis AM, Bueno SM. Role of *Salmonella enterica* exposure in Chilean Crohn's disease patients. *World J Gastroenterol* 2013; 19(35): 5855-5862 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5855.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5855

INTRODUCTION

Crohn's disease (CD) is an immunological bowel disorder of unknown etiology, characterized by chronic relapsing inflammation of the gastrointestinal tract[1]. CD is a complex and heterogeneous disorder[2], which is determined by, among other factors, the interaction between diverse environmental and genetic factors[3]. Studies on migrants have highlighted the importance of environmental factors in CD, and it is likely that there are important geographical differences[4,5]. Among the risk factors associated with CD are infections and smoking[6-12], and both are major public health problems[13-15] (http://www.ispch.cl/sites/default/files/Vigilancia_ Salmonella_spp_0). Preliminary studies have suggested a correlation between CD and infectious gastroenteritis[16,17]. A previous report showed that, in some populations, the incidence of CD increases over time after an acute gastroenteritis episode caused by *Salmonella enterica* (SE)[18]. However, a recent report did not find an association between SE and CD[19]. Different gene-environmental interactions and gene-gene interactions have been studied in CD and are frequently related to the *NOD2/CARD15* gene[20-25]. Polymorphisms in this gene have been the most consistent and significant genetic factor implicated in CD susceptibility[26]. However, studies of environmental-environmental interaction and their implications are scarce[27,28].

The reason for the possible association between SE and CD is unknown, but it is possible that mucosal immunity changes after SE exposure, promoting immune modification of the intestinal mucosa[29]. Thus, the intestinal mucosa may develop an enhanced inflammatory response when other factors affecting the immune response (genetic or environmental) are present. Moreover, the protein encoded by the *NOD2/CARD15* gene acts as an intracellular receptor that recognizes bacteria-derived molecules, including SE[30,31]. In addition, the clinical implications of SE in CD are unknown.

The aim of this study was to explore whether there is an association between SE exposure and CD and its implications in Chilean patients with CD.

MATERIALS AND METHODS

CD patients and clinical definitions

Ninety-four unrelated Chilean CD patients from CAREI (Active Cohort Registry of Inflammatory Bowel Disease) visiting a single inflammatory bowel disease (IBD) unit of a university referral center between December 2010 and June 2012 were included in this study. All CD patients gave written informed consent to participate in the study, and the local ethics committee approved the project. The study was performed in accordance with the principles stated in the declaration of Helsinki.

The diagnosis of CD was based on standard clinical, radiological, endoscopic, and histological criteria[32]. A complete clinical evaluation was performed at the initial visit, and all the important data of the clinical evolution of CD were obtained from a prospective clinical database of our unit. This evaluation included gender, age at diagnosis of CD, length of follow-up, location and behavior of CD, family history of IBD, previous appendectomy, smoking habit, oral contraceptive use, extra-intestinal manifestations of CD, and history of gastroenteritis caused by SE and/or vaccine for SE. The following characteristics were also documented: need for surgery or hospitalization for CD, use of steroids, use of immunosuppressants (azathioprine, 6-mercaptopurine and methotrexate) and use of anti-tumor necrosis factor (TNF) therapy.

Smokers were defined as patients who smoked more than seven cigarettes per week (one cigarette daily). A for-

mer smoker was defined as a patient who quit smoking at least 1 year before diagnosis. Nonsmokers were defined as patients who had never smoked or who smoke less than seven cigarettes per week[9]. The location and behavior of CD were determined according to X-ray and endoscopic findings; all patients underwent these procedures at least once at our center. The location and behavior of CD were defined according to criteria of the Montreal classification[2]; however, behaviors were registered as non-exclusive categories if intestinal stricturing occurred at a different time from the penetrating lesions; therefore, patients could belong to both categories. The severity of the disease was estimated as the appearance of stricturing or penetrating lesions or the need for immunosuppressants, anti-TNF therapy, hospitalization or surgery.

Control patients

Eighty-eight unrelated Chilean healthy blood donors, matched by sex and age, were studied as control subjects to determine if there were any differences in the frequency of exposure to SE, independent of *NOD2/CARD15* gene status. In addition, we studied the frequency of SE exposure in a sample of patients without CD who were visiting a respiratory disease unit at our university center. These patients were differentiated by their smoking status and matched by sex and age. Thus, we studied 90 patients who were distributed as follows: 30 smokers, 30 nonsmokers, and 30 former smokers. Smoking status was defined in the same way as in CD patients[9].

Determination of Salmonella exposure

For each subject, a venous blood sample was extracted to obtain serum, which was stored at -70 ℃ freezer until analysis. To evaluate previous exposure to *Salmonella*, serum samples were tested for the presence of anti-*Salmonella* lipopolysaccharide (LPS) antibodies [immunoglobulin-G type (IgG)] using an enzyme-linked immunosorbent assay (ELISA)[33]. The sensitivity and specificity of this methodology have been evaluated in a previous study[34]. Briefly, ELISA plates (Nalgene-Nunc®, Thermo Scientific, Waltham, MA, United States) were activated overnight at 4 ℃ with 500 ng of *Salmonella typhimurium* LPS (L7261-25MG SIGMA-ALDRICH, St. Louis, MO, United States) in 50 μL 0.1 mol/L bicarbonate buffer ($NaHCO_3$, Merck, Whitehouse Station, NJ, United States) and blocked with 100 μL of PBS-BSA 3% for 1 h at room temperature. Then, 50 μL of serum diluted in PBS at 1/64, 1/128, and 1/256 was added to each well and incubated for 2 h at room temperature. Next, three washes with 200 μL of PBS-Tween 0.05% were performed. After the washes, 50 μL of an IgG conjugated with peroxidase (clone G18-145, Becton Dickinson, Franklin Lakes, NJ, United States) was added to each well (diluted 1/2000 in PBS) and incubated for 2 h at room temperature. Three washes with PBS-Tween 0.05% were then performed, and the positive reaction was developed using 3-3'-5-5'-tetramethyl-benzidine at a final concentration 100 μg/mL (Sigma-Aldrich) as a colorimetric substrate. The enzymatic reaction was stopped with 2 mol/L H_2SO_4, and absorbance was recorded at 450 nm in an ELISA plate reader (Thermo Scientific). As a positive control, a group of subjects who had a previous history of gastroenteritis caused by SE or typhoid fever were included in this study and were used to standardize the methodology. As negative controls, a group of control subjects that had not been exposed to *Salmonella* were included, which comprised a serum of a pool of nine subjects up to 19 years old with no previous clinical history of gastroenteritis or typhoid fever. Both positive and negative controls were included in all the assays performed. Patients exposed to SE were defined as those patients with OD values higher than 2 SD over the value obtained for negative controls in the three dilutions tested in each determination. Those subjects not exposed to SE were all the other CD patients. As described before, this test allows the identification of patients that have been infected with either *Salmonella Typhimurium* or *Salmonella Enteritidis*[34]. These two serovars account for 80% of the cases of gastroenteritis caused by *Salmonella* in Chile (http://www.ispch.cl/sites/default/files/Vigilancia_Salmonella_spp_0.pdf).

NOD2/CARD15 genotyping

To detect *NOD2/CARD15* gene variants, genomic DNA from whole blood samples was isolated using standard molecular biology techniques. Specific sequences of exon 4 (missense mutation R702W), exon 8 (missense mutation G908R) and exon 11 (frameshift mutation L1007fsinsC) of the *Nod2/CARD15* gene were amplified by a polymerase chain reaction (PCR, using primers 5'- CCT TCA GAT CAC AGC AGC CTT C -3' and 5'- GGG ATG GAG TGG AAG TGC TTG -3' for exon 4; 5'- TCT AAG TCT GTA ATG TAA AGC CAC -3' and 5'- AGC TCC TCC CTC TTC ACC TGA -3' for exon 8, and 5'- CTG AGC CTT TGT TGA TGA GCT C -3' and 5'- ATT CTT CAA CCA CAT CCC CAT TC -3' for exon 11. PCR amplifications were performed in a MaxiGene Gradient Thermocycler (Axygen, Union City, CA, United States), using 250 ng of DNA, 1 nmol/mL of each primer, 0.2 mmol/L deoxynucleoside triphosphates, 1.5 mmol/L Magnesium Chloride and 50 U/mL of Taq DNA polymerase (Invitrogen, Carlsbad, CA, United States), using standard PCR amplification cycles. The PCR products obtained for exon 4 and exon 8 were digested with the restriction enzymes *Hpa* II and *Hha* I (New England Biolabs, Ipswich, MA, United States), and the digested PCR products were resolved by electrophoresis in 1% agarose gels containing 0.5 μg/mL ethidium bromide and visualized under a UV light transilluminator (UVP, Inc., Upland, CA, United States). PCR products obtained for exon 11 were gel purified and sequenced in an ABI 3100 automatic sequencer by Macrogen (http://www.macrogen.com). Frameshift mutations were analyzed for each sample, using the Vector NTI software (Invitrogen). CD patients positive for at least one of the

Table 1 Characteristics of Crohn's disease patients *n* (%)

Characteristic	*n* = 94
Gender	
Female	54 (57)
Age at diagnosis (yr)	
Median (range)	31 (9-80)
Early onset (< 40 yr)	61 (64.0)
Follow-up (yr)	
Median (range)	7.0 (2.0-34.0)
Smoking at diagnosis	31 (32.0)
History of gastroenteritis due to SE	5 (5.0)
Previous appendectomy	20 (21.0)
Disease localization	
Ileum	30 (31.9)
Ileocolonic	29 (30.8)
Colon	32 (31.9)
Upper gastrointestinal tract	3 (3.1)
Disease behavior	
Non-stricturing, non-penetrating	59 (62.7)
Stricturing	24 (25.5)
Penetrating	15 (15.9)
Perianal disease	23 (24.4)
Extraintestinal manifestation	49 (52.1)
Family history of IBD	14 (14.8)

SE: *Salmonella enterica*; IBD: Inflammatory bowel disease.

NOD2/CARD15 gene polymorphisms were categorized as gene variant carriers.

Statistical analysis

CD patients were compared to control subjects in relation to the presence of anti-SE IgG. Among CD patients, we compared those exposed to SE *vs* unexposed in relation to the most important clinical and genetic characteristics associated with CD. Categorical variables were compared using the Fisher's exact test. Continuous variables were expressed as the mean ± SD, and compared using Student's *t* test. A *P* value of less than 0.05 was considered to indicate statistical significance. Analysis was carried out using the StatView software package (SAS Institute Inc., Cary, NC, United States).

RESULTS

Patient population

The study population consisted of 94 patients with CD; 54 females and 40 males. Age at diagnosis was 35.5 ± 15.2 years. Mean follow-up was 9.0 ± 6.8 years, with a median of 7.0 years. Behavior of CD was inflammatory in 59 patients (62.7%), stricturing in 24 patients (25.5%) and penetrating in 15 patients (15.5%). Thirty cases (31.9%) had lesions in the terminal ileum, 29 (30.8%) were ileocolonic, 32 (34.0%) had colonic lesions and 23 (24.4%) had perianal disease. Five patients (5%) reported previous gastrointestinal infection caused by *Salmonella,* and only one patient (1%) had received a vaccine for *Salmonella*. Thirty-one CD patients (32.9%) were smokers, and seven (7.4%) were former smokers at diagnosis. The clinical characteristics of CD are shown in Table 1.

Table 2 Comparison between Crohn's disease patients exposed and those not exposed to *Salmonella enterica*

	IgG SE (+) (*n* = 16)	IgG SE (-) (*n* = 78)
Age at diagnosis (yr)	38.6 ± 15.6	34.9 ± 15.1
Female	6 (37.5)	48 (61.5)
Disease duration, years (yr)	11.2 ± 9.5	8.5 ± 6.1
Disease site		
Ileum	5 (31.2)	25 (32.0)
Ileocolonic	6 (37.5)	23 (29.4)
Colonic	5 (31.2)	27 (34.6)
Proximal	0 (0.0)	3 (3.8)
Perianal disease	1 (6.2)	22 (28.2)
Disease Behaviour		
Inflammatory	12 (75)	47 (60.2)
Stricturing	2 (12.5)	22 (28.2)
Penetrating	2 (12.5)	13 (16.6)
Family history of IBD	3 (18.7)	11 (14.1)
Active smokers	10 (62.5)	21 (26.9)[b]
Previous appendectomy	6 (37.5)	14 (17.9)
Oral contraceptive use	1 (6.2)	4 (5.1)
Extraintestinal involvement	7 (43.7)	42 (53.8)
Immunosuppressants use	11 (68.7)	55 (70.5)
Steroid use	13 (81.2)	67 (85.8)
Anti TNF therapy	1 (6.2)	7 (8.9)
Hospitalization due to IBD	13 (81.2)	58 (74.3)
Bowel Surgery	3 (18.7)	26 (33.3)
NOD2/CARD15 Variant	5 (31.2)	12 (15.3)

Data are expressed as absolute numbers (percentage) or mean ± SD. [b]*P* < 0.01 *vs* IgG *Salmonella enterica* (SE) (+). IBD: Inflammatory bowel disease; TNF: Tumor necrosis factor.

Exposure to SE and analysis of factors influencing exposure to SE

Sixteen CD patients (17%) were exposed to SE, as determined by the presence of anti SE-IgG in the serum. Among the 88 sex- and age-matched healthy blood donors, 15 patients (17%) were exposed to SE. There was no difference in exposure to SE between CD and control subjects (*P* = 0.8).

Comparison of the clinical characteristics between those who had been exposed to SE and those who had not, are shown in Table 2. With the exception of smoking, there were no significant differences in the clinical variables studied between the group of patients exposed to SE and those not exposed. Exposure to SE was significantly associated with cigarette smoking; in the group exposed to SE, 10 of 16 patients (62.5%) were active smokers compared to 21 of 78 patients (26.9%) in the group that was not exposed (*P* = 0.01). On the other hand, 10 of 31 smokers (32%) had exposure to SE compared with 5 of 56 nonsmokers (9%) and 1 of 7 former smokers (14%) (*P* = 0.01). No other factors were associated with exposure to SE. We also analyzed whether *NOD2/CARD15* gene variations were associated with exposure to SE, and the result was negative (*P* = 0.2, Table 2).

To assess whether increased exposure to SE was specific to CD patients who smoked, we determined the frequency of exposure to SE in smoking patients without CD. In these patients, there was no difference in exposure

to SE, defined by 3 of 30 smokers (10%) with detectable levels of anti-SE IgG compared to 5 of 30 non-smokers (16%) and 5 of 30 former smokers (16%), and the *P* value was non-significant ($P = 0.6$).

Age at diagnosis of CD, sex distribution, duration of CD, CD location, family history of IBD, and extraintestinal manifestations were similar in both groups of patients (Table 2), whereas other environmental clinical characteristics, such as use of oral contraceptives and previous appendectomy, were similar among carriers and non-carriers of anti-SE IgG (Table 2).

CD severity according to presence of anti-SE IgG

The prevalence of several indicators of disease severity, including stricturing or penetrating lesions, the need for immunosuppressants, anti-TNF therapy, hospitalization or surgery was similar in CD patients with and those without anti-SE IgG antibodies (Table 2).

NOD2/CARD15 genotype

Seventeen CD patients (18%) had at least one mutation of the *NOD2/CARD15* gene. All were heterozygous for these variants. The distribution of the three variants was as follows: 11 (11%) for R702W, one (1%) for G908R and five (5%) for L1007fsinsC. This frequency was higher than in control subjects, with 3 of 88 healthy blood donors (3%) displaying least 1 *NOD2/CARD15* gene mutation ($P = 0.003$).

DISCUSSION

This study evaluated the role of *Salmonella* in CD and its interactions with genetic (*NOD2/CARD15* gene) and environmental risk factors in Chilean patients. This is a step towards unraveling the importance of environmental factors in CD and their association with other risk factors in a new population. In CD, an important part of the disease pathogenesis could be related to environmental factors and their interaction with other patient factors[1,3,11,22,27]. Our study showed no difference in the level of previous exposure to *Salmonella* between CD patients and controls. On the other hand, we observed a striking correlation between smoking and exposure to SE in CD, independent of *NOD2/CARD15* gene variants, implying a particular pattern of environment-environment interaction.

The role of microbes in CD is supported by the fact that most mouse models of IBD develop colitis only in the presence of intestinal bacteria[35], and several human studies have shown remissions in CD patients after antibiotic therapy[36]. A growing body of evidence suggests a correlation between CD and infectious gastroenteritis[16,17] (http://www.ispch.cl/sites/default/files/Vigilancia_ Salmonella_spp_0.pdf). Although a previous study implicated *Salmonella* as a risk factor for CD, in which patients with gastroenteritis caused by *Salmonella* had a higher risk for developing IBD compared to age- and sex-matched controls[18], our study showed no difference in previous exposure to *Salmonella* between CD patients and controls. The design of our study differs from other studies because the diagnostic criterion was based on a cross serological test to determine previous exposure to SE using the presence of anti-*Salmonella* IgG. Given the relatively low rate of history of previous typhoid fever or gastroenteritis caused by SE in our CD patient population, it is possible that independent factors of gastrointestinal infections may be more relevant in our population, including non-*NOD2/CARD15* genes, given the low frequency of mutations in this gene in our CD patients. However, our findings agree with a recent report that did not find an association between SE and CD, and this study strongly suggests that the positive associations observed in the earlier studies were the result of a detection bias[19]. The study of Jess *et al*[19] showed that the temporal risk patterns for IBD are not different following negative and positive stool tests for SE. This observation strongly suggests that increased occurrence of *Salmonella* around the time of diagnosis results from detection bias resulting from increased rates of stool testing. Moreover, the occurrence of *Salmonella* infection in patients with nonspecific gastrointestinal symptoms compatible with CD could represent a "by chance" finding and should not exclude the patients from subsequent clinical examination if gastrointestinal symptoms persist[19].

In other autoimmune diseases, such as rheumatoid arthritis (RA), smoking is a well-established environmental risk factor[15,37,38]. In CD, smoking is also a major risk factor, and it is associated with several complications over the course of CD[9,10,39]. A meta-analysis supports the view that current smoking is associated with a significantly higher risk of CD[40]. Recent studies have reinforced the importance of smoking, suggesting that studies of risk factors for IBD should be stratified for smoking behavior, especially in cohorts of limited sample size, as in the current study[41].

We found a significant association between *Salmonella* exposure and active smoking in a group of Chilean CD patients. These two factors may have an additive effect on the development of the disease in a subset of CD patients. To date, there is no clear biological explanation for why smoking is associated with an increased risk of CD[42]. In smokers with CD, an increased immune response against *Salmonella* could be developed, based on a greater presence of anti-*Salmonella* IgG. SE may produce a change in mucosal immunity[29], and this change could be exacerbated in smoking patients. This could lead to tissue damage and the onset of IBD in susceptible hosts. *Salmonella* triggers an inflammatory response categorized as Th1[43], and given that intestinal immune response in CD is classically recognized as Th1, it is conceivable that invasive bacteria, such as *Salmonella,* could trigger this abnormal intestinal immune reaction. In addition, inflammation is required by SE to colonize intestinal mucosa and compete with resident microflora[44]. In experimental studies, prolonged exposure to concentrated smoke leads to decreased expression and activity of the anti-inflam-

matory enzyme heme oxygenase-1[45], and an increase in cell autophagy in the bowel[46]. It is possible that chronic smoking may cause a pro-inflammatory state, and *Salmonella* infection could be facilitated in smoking patients. *Salmonella* infection and tobacco consumption are a major public health problem in Chile and worldwide[9,13-15] (http://www.bcn.cl/carpeta_temas/tem as_portada.2006-09-25.0806013222/documentospdf-sobre-obesidad/VIGIA20.pdf and http://www.redsalud.gov.cl/portal/docs/page/minsalcl/g_home/submenu_portada_2011/ens2010.pdf), and there should be a greater emphasis on the importance of food safety and smoking avoidance.

Notably, we did not observe any association with a higher severity in patients exposed to SE compared to those not exposed, defined as the presence of stricturing or penetrating lesions or the need for immunosuppressants, anti-TNF therapy, hospitalization or surgery.

A limitation of our study is the relatively small sample population; therefore, future larger studies will be needed to confirm the relationship between smoking and infection with SE. However, the absence of this association in a control sample without CD supports the notion that this is a specific feature of CD patients.

In conclusion, our study found no association between exposure to SE and CD in a new well-defined population of Chilean CD patients. We observed a positive correlation between SE exposure and cigarette smoking in patients with CD, but not with disease severity. This research more precisely defines the real role of *Salmonella* exposure, an important environmental factor in CD, and how risk factors combine to trigger CD in a given patient.

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COMMENTS

Background

Different environmental factors have been studied in Crohn's disease (CD). An important environmental factor that has been implicated as a possible trigger of CD is infectious gastroenteritis. There is a major controversy concerning the role of *Salmonella enterica* (SE) as a risk factor for CD. It is possible that there are important geographical differences; however, to date, there are still no data addressing the interactions between SE exposure and other environmental factors and the clinical implications of these interactions in CD.

Research frontiers

Different gene-environment and gene-gene interactions have been studied in CD. However, environment-environment interaction studies and their clinical implications are scarce. There may be important ethnic variations. The focus of this research was to establish the real importance of an environmental factor as infectious gastroenteritis caused by SE in Chilean CD patients.

Innovations and breakthroughs

Results of previous studies on the role of SE in CD have been controversial. The present study was designed to evaluate the role of SE in CD and its interactions with genetic and environmental risk factors in Chilean patients. The authors determined previous exposure to SE defined by the presence of anti-*Salmonella* IgG. Although their study showed no difference in previous exposure to *Salmonella* between CD patients and controls, they observed that smoking was associated with exposure to SE in CD. If they analyze a key element, such as clinical implication of SE exposure, they did not observe a correlation between SE exposure and CD severity. This research more precisely defines the role of *Salmonella* infection, an important environmental factor, in CD and how risk factors combine to trigger CD in a given patient.

Applications

The study results suggests that SE exposure is not associated with CD; however, the authors observed that smoking was associated with exposure to SE in Chilean CD patients, independent of other factors, implying a particular pattern of environment-environment interaction.

Terminology

Gene-gene, gene-environment, or environment-environment interactions: In complex diseases, such as CD, there are different implications or consequences for the disease when two factors (gene and/or environmental) are present in the same patient.

Peer review

This paper represents a considerable amount of good work, and it is a well-done and controlled study. It is a study in a well-defined population. This manuscript aimed to study the association between exposure to SE and CD. In addition, they also analyzed the involvement of cigarette smoking. They showed no association for the SE status and CD, but positive association between SE and smoking in CD.

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BRIEF ARTICLE

Modulation of individual components of gastric motor response to duodenal glucose

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Abstract

AIM: To evaluate individual components of the antro-pyloro-duodenal (APD) motor response to graded small intestinal glucose infusions in healthy humans.

METHODS: APD manometry was performed in 15 healthy subjects (12 male; 40 ± 5 years, body mass index 26.5 ± 1.6 kg/m^2) during four 20-min intraduodenal infusions of glucose at 0, 0.5, 1.0 and 1.5 kcal/min, in a randomised double-blinded fashion. Glucose solutions were infused at a rate of 1 mL/min and separated by 40-min "wash-out" period. Data are mean ± SE. Inferential analyses are repeated measure analysis of variance with Bonferroni post-hoc testing.

RESULTS: At 0 kcal/min frequency of pressure waves were: antrum (7.5 ± 1.8 waves/20 min) and isolated pyloric pressure waves (IPPWs) (8.0 ± 2.3 waves/20 min) with pyloric tone (0.0 ± 0.9 mmHg). Intraduodenal glucose infusion acutely increased IPPW frequency ($P < 0.001$) and pyloric tone ($P = 0.015$), and decreased antral wave frequency ($P = 0.007$) in a dose-dependent fashion. A threshold for stimulation was observed at 1.0 kcal/min for pyloric phasic pressure waves ($P = 0.002$) and 1.5 kcal/min for pyloric tone and antral contractility.

CONCLUSION: There is hierarchy for the activation of gastrointestinal motor responses to duodenal glucose infusion. An increase in IPPWs is the first response observed.

Key words: Glucose; Gastrointestinal motility; Pyloric; Antral; Duodenum; Manometry; Motor activity; Blood glucose

Core tip: Antro-pyloro-duodenal manometry was performed in 15 healthy subjects. Subjects were randomly given 20 min intraduodenal infusions of glucose at 0, 0.5, 1.0 and 1.5 kcal/min. Intraduodenal glucose infusion acutely increased isolated pyloric pressure wave

frequency and pyloric tone and decreased antral wave frequency in a dose-dependent fashion. A threshold for stimulation was observed at 1.0 kcal/min for pyloric phasic pressure waves and 1.5 kcal/min for pyloric tone and antral contractility. These data suggest that there is hierarchy for the activation of gastrointestinal motor responses to small intestinal glucose stimulation.

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INTRODUCTION

Gastric emptying of liquid nutrient is regulated at approximately 2-3 kcal/min by antro-pyloro-duodenal (APD) motor activity[1,2]. Nutrient in the small intestine stimulates receptors and initiates a feedback loop that affects motility of individual components of the APD area[3]. These motor changes include antral suppression[1], and stimulation of phasic and tonic pyloric contractions[4,5].

The precise APD motor response to nutrient probably depends in part on the macronutrient composition of the "meal". Recent data suggest that the "doses" of lipid nutrient initiating these motor changes in health are much less than previously recognised[6]. Likewise small intestinal carbohydrate infusions at rates that are within normal gastric emptying rates have marked effects on the APD unit[7]. However, the specific threshold and/or hierarchy of the APD response to nutrient stimulation are unknown. The aim of this study was to assess the responses of the distal stomach to graded small intestinal nutrient stimulation in health.

MATERIALS AND METHODS

Subjects

Studies were performed in fifteen healthy volunteers [male:female, 12:4; age: 40 ± 5 years; body mass index (BMI): 26.5 ± 1.6 kg/m^2]. Subjects were screened and excluded if they were diabetic, pregnant or breast feeding, had previous gastrointestinal surgery, a history of gastrointestinal disease or taking medications known to alter gastrointestinal motility. None of the subjects regularly smoked tobacco or drank more than 20 g of alcohol per day.

The protocol was approved by the research ethics committee of the Royal Adelaide Hospital, and each subject gave written informed consent prior to the commencement of the study.

Measurement techniques

Multi-lumen perfusion manometry: APD motility was assessed by a 100-cm multi-lumen perfusion manometric assembly (outer diameter 3.5 mm; Mui Scientific, Ontario, Canada). The assembly incorporated 15 pressure recording channels (side-holes spaced 1.5 cm apart), with a 4.5 cm sleeve-sensor, and an infusion port. Correct placement of the sleeve across the pylorus was determined using continuous measurement of the antro-duodenal transmucosal potential difference (TMPD) gradient[8]. The assembly was positioned so that five side holes (A1-A5) were located in the gastric antrum and seven in the proximal duodenum (Figure 1). The infusion port was located at the catheter tip to enable the delivery of enteral feed directly into the duodenum 9 cm distal to the pylorus. Thirteen manometric lumina were perfused with degassed water at a rate of 0.04 mL/min except for the sleeve perfused at a rate of 0.15 mL/min. To monitor TMPD two channels on either end of the sleeve were perfused with degassed 0.9% saline. Pressure and TMPD data were recorded on a computer using purpose written software program (Medical Measurement Systems, Enschede, The Netherlands)[8].

Blood glucose concentration: As hyperglycaemia has a major impact on gastric motility[9], blood glucose concentrations were measured using a portable glucometer (Precision Plus, Abbott Laboratories, Bedford, United States) every 20 min throughout the study.

Protocol

Subjects were studied in the gastrointestinal motility laboratory of the Royal Adelaide Hospital after an overnight fast. The manometric catheter was inserted into an anaesthetised nostril and passed into the stomach. The catheter passed into the duodenum assisted by spontaneous peristalsis. A cannula was inserted into an antecubital vein for blood sampling.

Each subject received intraduodenal infusions of 50% glucose solution (Pharmalab NSW Australia) diluted in water at: (1) 0.5 kcal/min; (2) 1 kcal/min; (3) 1.5 kcal; and (4) 0 kcal/min (0.9% saline only). Each solution was prepared in separate 20 mL syringes by a study investigator who was not involved in the data analysis and infused at a rate of 1 mL/min. Randomisation of glucose load was computer generated. Each syringe was then covered by the investigator preparing the syringes and labelled according to the randomisation schedule. The syringe was connected to the manometric catheter using opaque minimal volume extension tubing to ensure blinding of the research staff.

Following correct positioning of the catheter sleeve across the pylorus, a 20 min fasting period commenced. At the end of the fasting period the scheduled load was infused directly into the duodenum, *via* a volumetric syringe driver [Terumo Syringe Pump (STC-523), Medtel Australia], followed by a 40 min "washout" period of 0.9% saline (1 mL/min). A similar schedule was followed for all glucose loads. Blood samples were taken every 20 min throughout the study period to measure blood glucose

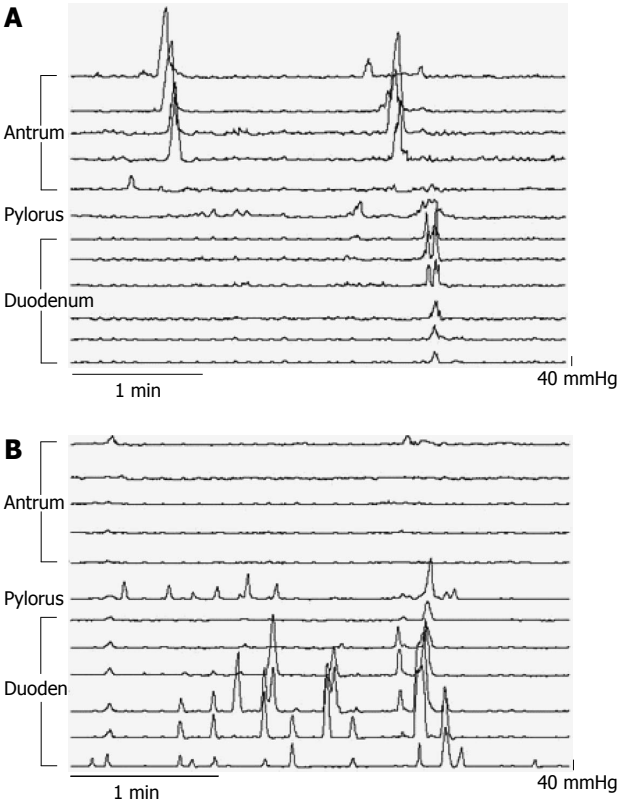

Figure 1 Representative trace of intraluminal motility at (A) 0 kcal/min and at (B) 1.5 kcal/min. Occlusive contraction waves commence in the antrum and propagate through the duodenum. At 1.5 kcal/min isolated pyloric pressure waves occurred more frequently and a reduction in propagated antral contractility was observed.

concentrations.

Data analysis

Manometric data were imported into Acqknowledge 3.2.7 and were analysed manually. The frequencies of APD pressure waves were determined as previously described[8]. In brief, pressure waves were included in the analysis when a rise in intraluminal pressure was greater than the minimum amplitude over the appropriate time-period and when the assembly was positioned correctly according to established TMPD criteria. Migrating motor complex (MMC) phase Ⅲ activity associated pressure waves were considered to be representative of fasting motor patterns and were counted as zero for the period of phase Ⅲ activity. Antral phase Ⅲ MMC activity was defined as rhythmic pressure wave activity occurring at a maximum frequency (three pressure waves per minute) for at least one minute with a temporal relationship with duodenal activity. Duodenal phase Ⅲ MMC was defined as a maximum frequency of 10-12 pressure waves/min for at least 2 min[8].

A pressure wave in the antrum and pylorus was defined as a pressure rise of 10 mmHg or more from baseline and lasting between 6.1 and 20 s[10]. Isolated pyloric pressure waves (IPPWs) were defined as pressure waves at least 10 mmHg amplitude recorded only in the sleeve channel[5]. A duodenal PW was defined as a pressure rise of 6 mmHg or more from baseline and lasting between 0.8 and 7 s[10]. Change in pyloric tone (basal pyloric pressure) was calculated as the difference in baseline pressure in the sleeve sensor from the duodenum[11] at 4-min intervals and presented as mean over 20 min.

Statistical analysis

Data are presented as mean ± SE. Repeated-measures analysis of variance (RM-ANOVA) were used to test for effects on pressure wave activity and pyloric tone of different caloric loads. Residuals were normally distributed and, furthermore, analyses using the equivalent non-parametric test (Friedman) remained significant. On testing there was no order effect apparent. Differences at the level of $P < 0.05$ were considered significant and allowed post-hoc comparison between loads which were corrected according to Bonferroni adjustment.

RESULTS

All subjects tolerated the study without adverse symptoms or effects.

Motility

An example of a manometric trace at two different loads is shown in Figure 1.

Antral pressure waves: The effect of glucose loads on antral pressure wave activity is shown in Figure 2A. Increasing the caloric load had an effect on antral wave frequency ($P = 0.007$) with marked attenuation of antral pressure wave activity at 1.5 kcal/min, when compared to 0 kcal/min (0 kcal/min: 7.5 ± 1.8 waves/20 min *vs* 1.5 kcal: 2.8 ± 1.3 waves/20 min; $P = 0.007$).

IPPWs: The effects of glucose loads on IPPW activity are shown in Figure 2B. The frequency of IPPWs were affected by caloric load ($P < 0.001$) with a substantial increase in pressure waves occurring with increasing nutrient (0 kcal/min: 8.0 ± 2.3 waves/20 min *vs* 1.0 kcal: 25.9 ± 3.7 waves/20 min; $P = 0.002$). The increasing frequency of IPPW during glucose infusion occurred in a dose dependent fashion with 1.0 kcal/min the observed threshold to stimulate the pylorus (0 kcal/min: 8.0 ± 2.3 waves/20 min *vs* 0.5 kcal/min: 14.2 ± 2.7 waves/20 min; $P = 0.294$; but 0.5 kcal/min 14.2 ± 2.7 waves/20 min *vs* 1.0 kcal: 25.9 ± 3.7 waves/20 min; $P = 0.037$).

Duodenal pressure waves: The effects of glucose loads on duodenal wave activity are shown in Figure 2C. There was a difference between treatments over time in duodenal motor wave activity with different caloric loads ($P = 0.012$). However, post-hoc testing did not reveal a difference between the individual loads (0 kcal/min: 24.4 ± 4.7 waves/20 min *vs* 34.7 ± 4.9 waves/20 min, $P = 0.22$; and 0.5 kcal/min 21.7 ± 3.3 waves/20 min *vs* 34.7 ± 4.9 waves/20 min, $P = 0.058$).

Pyloric tone: The effects of glucose loads on pyloric

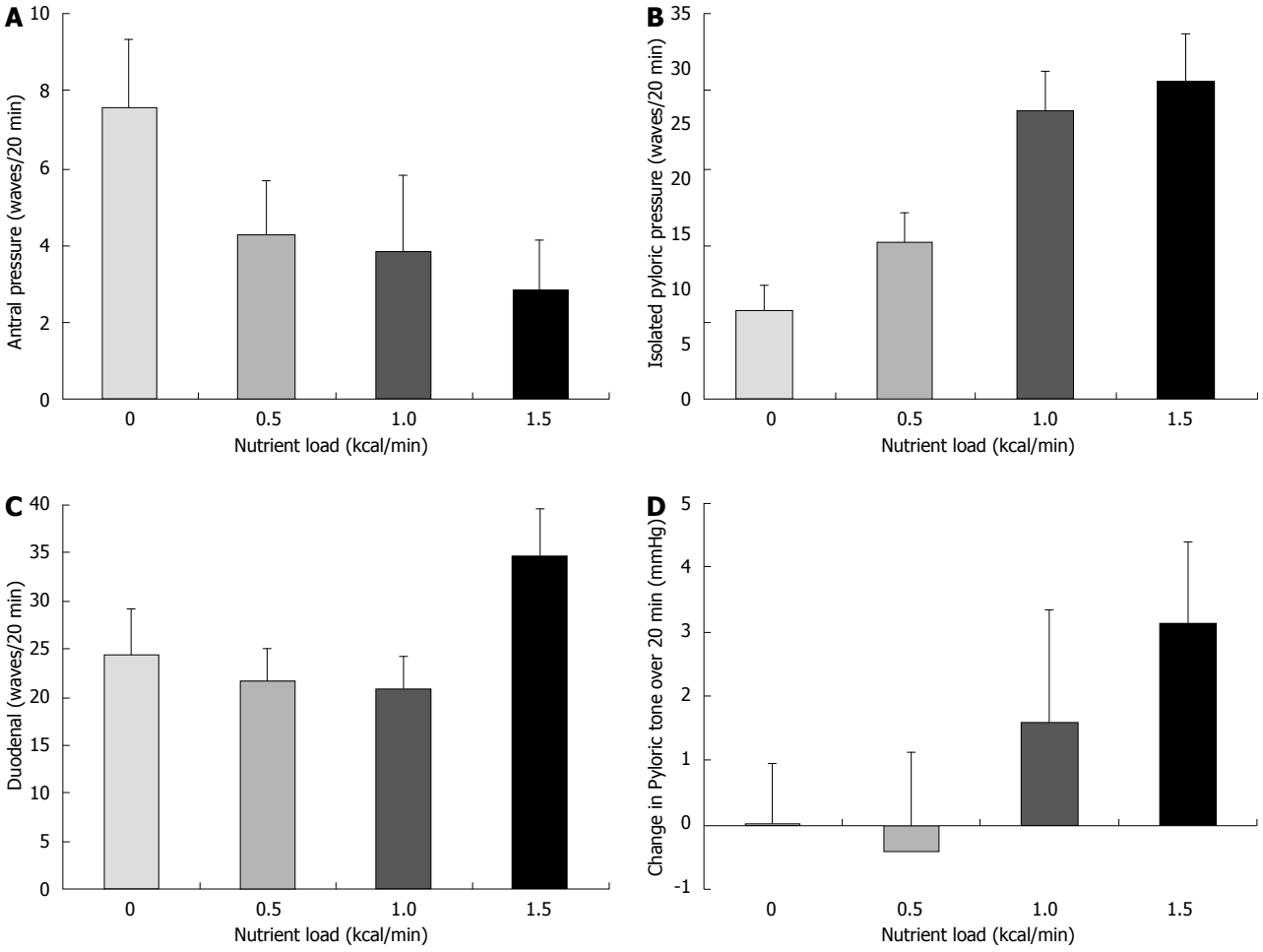

Figure 2 Antral, isolated pyloric, duodenal pressure waves and pyloric tone at differing caloric loads. A: Reduction in antral motility occurred with increasing glucose load with marked attenuation of antral pressure wave activity at 1.5 kcal/min, when compared to 0 kcal/min (0 kcal/min: 7.5 ± 1.8 waves/20 min *vs* 1.5 kcal: 2.8 ± 1.3 waves/20 min; $P = 0.007$); B: The frequency of isolated pyloric pressure waves increased with increasing caloric load. The number of waves/20 min were statistically different between 0 and 1.0 kcal/min (0 kcal/min: 8.0 ± 2.3 waves/20 min *vs* 1.0 kcal/min: 25.9 ± 3.7 waves/20 min; $P = 0.002$); C: The nutrient load did not effect duodenal wave frequency when comparing 0 kcal/min to 1.5 kcal/min (0 kcal/min: 24.4 ± 4.7 waves/20 min *vs* 1.5 kcal/min: 34.7 ± 4.9 waves/20 min; $P = 0.22$); D: Pyloric tone increased in response to increasing caloric loads ($P = 0.015$). Between 0.5 and 1.5 kcal/min the difference was significant (0.5 kcal/min: -0.4 ± 1.1 mmHg *vs* 1.5 kcal/min: 3.1 ± 1.3 mmHg; $P = 0.008$).

tone are shown in Figure 2D. There was an observed difference between treatments in pyloric tone ($P = 0.015$). The difference between 0 and 1.5 kcal/min was significant prior ($P = 0.035$), but not following Bonferroni adjustment (0.0 ± 0.9 *vs* 3.12 ± 1.3; $P = 0.207$). However, the difference remained significant between 0.5 and 1.5 kcal/min (-0.4 ± 1.1 *vs* 3.1 ± 1.3; $P = 0.008$).

Blood glucose concentrations

Blood glucose concentrations were similar prior to commencing each infusion (0, 0.5, 1.0 and 1.5 kcal/min: 6.5 ± 0.3 mmol/L, 5.9 ± 0.2 mmol/L, 5.8 ± 0.3 mmol/L *vs* 5.7 ± 0.2 mmol/L; $P = 0.625$) and at the completion of the infusion (5.9 ± 0.2, 5.9 ± 0.2, 6.4 ± 0.3 *vs* 6.2 ± 0.2; $P = 0.079$).

DISCUSSION

The major finding of this study is that, in health, there is a hierarchical response in the APD motor area to increasing glucose loads. The hierarchical response is graded, with initial stimulation of IPPWs and then inhibition of antral activity and an increase in pyloric tone. An increase in duodenal pressure wave frequency also occurred with a caloric load of 1.5 kcal/min.

In health, gastric emptying of nutrient liquid is regulated by the distal stomach which is affected by nutrient stimulating receptors in the small intestine[2]. Pilichiewicz *et al*[6] showed that intraduodenal infusions of as little as 0.25 kcal/min of lipid emulsion (10% intralipid) attenuated antral motility and increased pyloric phasic pressure waves. The same authors also showed that glucose at 1 kcal/min for 120 min reduced antral wave frequency but an increase in IPPWs only occurred at 4 kcal/min[7]. Pressure waves isolated to the pylorus are an integral component of the APD response to duodenal nutrient, and may be the most important mechanism to slow gastric emptying[4]. In addition, pyloric pressure waves assist

with the mixing of chyme[12] and initial stimulation of IPPWs prior to effects on other components of the APD unit should assist with trituration. Accordingly, we hypothesised that APD motor function would be affected by carbohydrate load in a hierarchical fashion and, given their substantial importance, IPPWs would occur early. It was anticipated that the magnitude of effect may be, relatively, small and the protocol was designed to detect small differences. The carbohydrate loads chosen were around 1 kcal/min[7], which were considered physiologically relevant, the sample size was increased compared to previous studies[6,7] and the study was undertaken on a single day to minimise intrasubject variability. Lastly, the infusion periods were limited to 20 min as "adaptation" to small intestinal caloric loads has been reported during prolonged infusions[13] and if this occurred, it would have reduced the likelihood of detecting a true difference. This study shows that modulation of each component of the APD unit is hierarchical and dependent on caloric load; initially resistance to trans-pyloric flow occurs with IPPWs and, subsequently, antral propulsive force decreases. The implication of this finding is that small intestinal delivery of nutrient, even within so-called "normal" gastric emptying rates has a substantial effect on APD motor patterns.

Duodenal phasic activity is characterised by irregular motor patterns with both antegrade and retrograde pressure wave sequences[14]. These contraction wave sequences commonly propagate only over a short distance causing intermittent and bidirectional flow of chyme (to aid mixing of chyme and exposure of chyme to luminal receptors). However, more prolonged antegrade wave activity is required for aboral movement of chyme and it has been previously reported that increasing nutrient load decreases the frequency of the sequences[7,15]. In contrast, we detected a strong trend to increased duodenal activity with increasing loads. This may reflect a chance finding or the relationship between nutrient load and duodenal activity is non-linear, with initial small increments in load increasing frequency of contractions and above a certain threshold (perhaps 1.5 kcal/min) a reduction in duodenal wave frequency occurs.

The proposed mechanisms underlying modulation of the APD unit are neural and hormonal. Fone and colleagues showed that stimulation of phasic pyloric pressure waves during intraduodenal glucose at 2.4 kcal/min are mediated *via* ascending enteric nerves and ACh-stimulation of muscarinic receptors[16]. Both cholecystokinin and glucagon-like peptide-1 are secreted, albeit temporarily, in response to 1 kcal/min of intraduodenal glucose[17] and endogenous secretion of these hormones are known to have substantial effects on APD motility and, thereby, gastric emptying[18-21].

While this study was undertaken in health, implications of these data for pathological conditions can be speculated upon. Gastric emptying is frequently slowed with healthy aging and in conditions such as diabetes and critical illness[22-24]. This slowing has been attributed in part to "hypersensitivity" to small intestinal nutrient, which appears to be, at least in part, hormonally-mediated *via* hormones such as cholecystokinin[25]. These data in health suggest the hypothesis that the process of aging or the effects of critical illness exacerbates this hierarchical sensitivity. Further study in this area would be of interest.

The limitations of this study should be recognised. Only occlusive pressure waves are detected by manometry. Non-occlusive antral pressure waves and/or non-peristaltic flow may also have been affected[26]. However pyloric closure prevents transpyloric flow. It would be of interest to repeat this study using measurement techniques that detect non-occlusive antral pressure waves. In addition, the duodenal nutrient infusion was non-pulsatile and short term (20 min). However, the feedback mechanism is the same whether the intra-duodenal nutrient is infused in a pulsatile or non-pulsatile fashion[27]. The aim of this study was only to measure the acute response to duodenal nutrient infusion and as motor responses adapt[13], particularly at lower loads[17], the response during more prolonged stimulation at these loads could be different. It should also be recognized that blood glucose concentrations were not clamped and hyperglycemia, even within the physiological range, affects APD motor responses and gastric emptying[28-30]. However glucose concentrations were similar despite the differing loads, suggesting that this is unlikely to explain the adaptive response observed.

In summary, glucose loads as low as 1 kcal/min infused into the duodenum initiate APD motor responses that will slow gastric emptying. The acute APD motor response to an intra-duodenal carbohydrate load is hierarchical and "dose" dependant, with an increased frequency of IPPW, followed by a decrease in antral wave frequency.

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COMMENTS

Background

Emptying of liquid nutrient from the stomach is regulated by antro-pyloro-duodenal (APD) motor activity. Nutrient within the small intestine initiates a feedback loop that affects motility of the APD area. Recent data suggest that the "doses" of lipid nutrient initiating these motor changes in health are much less than previously recognised. Likewise small intestinal carbohydrate infusions at rates that are within normal gastric emptying rates have marked effects on the APD unit. However, the specific threshold and/or hierarchy of the APD response to nutrient stimulation are unknown. The aim of this study was to assess the responses of the distal stomach to graded small intestinal nutrient stimulation in health.

Research frontiers

The specific threshold and/or hierarchy of the APD response to nutrient stimulation are unknown.

Innovations and breakthroughs

This study shows that modulation of each component of the APD unit is hierarchical and dependent on caloric load; initially resistance to trans-pyloric flow oc-

curs with isolated pyloric pressure waves and, subsequently, antral propulsive force decreases.

Applications

The implication of this finding is that small intestinal delivery of nutrient, even within so-called "normal" gastric emptying rates has a substantial effect on APD motor patterns.

Terminology

APD manometry is a technique used to measure luminal occlusive contractions, which result in peristaltic flow.

Peer review

This is a continuum of authors' previous works and tested a role of diet in the duodenum in the stimulation of the APD motor response. It's worth to publish on this journal as a brief report or communication.

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BRIEF ARTICLE

ITGA1 polymorphisms and haplotypes are associated with gastric cancer risk in a Korean population

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Abstract

AIM: To evaluate the association between the genetic polymorphisms and haplotypes of the *ITGA1* gene and the risk of gastric cancer.

METHODS: The study subjects were 477 age- and sex-matched case-control pairs. Genotyping was performed for 15 single nucleotide polymorphisms (SNPs) in *ITGA1*. The associations between gastric cancer and these SNPs and haplotypes were analyzed with multivariate conditional logistic regression models. Multiple testing corrections were carried out following methodology for controlling the false discovery rate. Gene-based association tests were performed using the versatile gene-based association study (VEGAS) method.

RESULTS: In the codominant model, the ORs for SNPs *rs2432143* (1.517; 95%CI: 1.144-2.011) and *rs2447867* (1.258; 95%CI: 1.051-1.505) were statistically significant. In the dominant model, polymorphisms of *rs1862610* and *rs2447867* were found to be significant risk factors, with ORs of 1.337 (95%CI: 1.029-1.737) and 1.412 (95%CI: 1.061-1.881), respectively. In the recessive model, only the *rs2432143* polymorphism was significant (OR = 1.559, 95%CI: 1.150-2.114). The C-C type of *ITGA1* haplotype block 2 was a significant protective factor against gastric cancer in the both codominant model (OR = 0.602, 95%CI: 0.212-0.709, P = 0.021) and the dominant model (OR = 0.653, 95%CI: 0.483-0.884). The *ITGA1* gene showed a significant gene-based association with gastric cancer in the VEGAS test. In the dominant model, the A-T type of *ITGA1* haplotype block 2 was a significant risk factor (OR = 1.341, 95%CI: 1.034-1.741). SNP *rs2447867* might be related to the severity of gastric epithelial injury due to inflammation and, thus, to the risk of developing gastric cancer.

CONCLUSION: *ITGA1* gene SNPs *rs1862610*, *rs2432143*, and *rs2447867* and the *ITGA1* haplotype block that includes SNPs *rs1862610* and *rs2432143* were significantly associated with gastric cancer.

Key words: Integrin; *ITGA1*; Gastric cancer; Polymorphism; Haplotype

Core tip: There are few studies addressing the role of the integrin α 1 subunit in the development of gastric cancer. To the best of our knowledge, this study is the first to show that *ITGA1* gene single nucleotide polymorphisms and haplotypes are associated with gastric cancer risk.

Yim DH, Zhang YW, Eom SY, Moon SI, Yun HY, Song YJ, Youn SJ, Hyun T, Park JS, Kim BS, Lee JW, Kim YD, Kim H. *ITGA1* polymorphisms and haplotypes are associated with gastric cancer risk in a Korean population. *World J Gastroenterol* 2013; 19(35): 5870-5876 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5870.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5870

INTRODUCTION

Gastric cancer is the second most common cancer in South Korea and represents the second leading cause of cancer death for both men and women worldwide[1,2]. Approximately one million new cases of stomach cancer are estimated to have occurred (989000 cases, 7.8% of the total), currently making it the fourth most common malignancy in the world, following cancers of the lung, breast and colo-rectum[2]. Epidemiological studies have provided evidence that a high intake of salt and nitrite-rich foods and *Helicobacter pylori* (*H. pylori*) infection are associated with a high incidence of gastric cancer in South Korea[3-7].

The risk of developing gastric cancer is estimated to be increased 2-6 fold in patients with *H. pylori* infection[8]. The risk of gastric cancer among individuals infected with *H. pylori* is influenced by bacterial virulence. The most widely studied *H. pylori* virulence factors are the *cag* (cytotoxin-associated gene) antigens[9]. Compared to individuals infected with *cagA*-negative *H. pylori* strains, those infected with *cagA*-positive *H. pylori* strains show a higher risk of developing gastric cancer[10]. To introduce *cagA* into host cells, the *cagL* protein of *H. pylori* binds to integrins on the basolateral surface of gastric epithelial cells[11,12].

Integrins are members of a family of heterodimeric cell-surface proteins that mediate cell-matrix and cell-cell interactions. The 18 integrin α-subunits and 8 β-subunits together form at least 25 different integrins[13]. Integrins mediate signaling events that are essential for stable cell adhesion, spreading, migration, survival, proliferation and differentiation. Several integrins, including α1β1, bind to extracellular matrix proteins present in the basal membrane of mature vessels[14,15]. The tumor progression and metastasis of various cancers are associated with integrins[16,17].

The *ITGA1* gene, located on chromosome 5q11.2, encodes the integrin α1 subunit, which is involved in the adhesion of gastric cancer cells to the peritoneum. The adhesion of integrin α1-positive gastric cancer cells to the extracellular matrix is a critical process in peritoneal dissemination[18,19]. There are few studies addressing the roles of integrins in the development of gastric cancer. An association with an increased risk of gastric cancer has only been reported for the *ITGA2* C807T polymorphism in a Chinese population[20]. As the level of integrin α1β1 is up-regulated in association with inflammation of the gastrointestinal tract mucosa, which is the first step in gastric carcinogenesis[21], it is possible that the integrin α1 subunit plays an important role in gastric cancer development.

The purpose of this study was to evaluate the association between the genetic polymorphisms and haplotypes of the *ITGA1* gene and the risk of gastric cancer.

MATERIALS AND METHODS

Study subjects

This subjects included in this study consisted of 477 newly diagnosed gastric cancer patients and an equal number of age- (within 3 years) and sex-matched controls. The diagnoses of the gastric cancer patients were all histologically confirmed at Chungbuk National University Hospital and Eulji University Hospital, which are located in a geographically central region of South Korea. Controls were selected from individuals receiving routine medical examinations in these hospitals, and individuals with a previous diagnosis of any type of cancer were excluded. Trained interviewers used a structured questionnaire including questions about demographic factors, smoking habits, alcohol consumption and dietary habits to interview all subjects who provided written informed consent. Peripheral blood samples were collected from all subjects. This study was approved by the institutional review boards of Chungbuk National University Hospital, South Korea (IRB No. 2011-09-071).

Selection of single nucleotide polymorphisms in ITGA1

At the International HapMap Project website (http://hapmap.ncbi.nlm.nih.gov/), tag SNPs were selected using a cut-off minimum minor allele frequency in the JPT population of 0.05 and pairwise tagging (r^2 = 1-0.8). SNPs that significantly deviated from Hardy-Weinberg equilibrium were discarded.

Genomic DNA was extracted from whole blood using the QuickGene-810 nucleic acid isolation system (Fujifilm, Tokyo, Japan) and the QuickGene DNA Whole Blood Kit (Kurabo, Osaka, Japan), in accordance with the manufacturer's instructions. DNA was stored at 4 ℃ until use. SNP genotyping was performed using a GoldenGate Genotyping Assay with VeraCode technology (Illumina, San Diego, CA, United States). A custom GoldenGate assay was designed for the analysis of the selected SNPs in the *ITGA1* gene. Those SNPs were then assessed for suitability for the GoldenGate genotyping platform, and

Table 1 Characteristics of the study subjects *n* (%)

Variables	Controls (*n* = 477)	Cases (*n* = 477)	OR (95%CI)
Age (yr) mean ± SD	57.8 ± 10.2	58.7 ± 9.9	
Sex			
Males	301 (63.1)	301 (63.1)	
Females	176 (36.9)	176 (36.9)	
Smoking status			
Non-smokers	225 (47.6)	194 (41.0)	1.00 (reference)
Smokers	248 (52.4)	279 (59.0)	1.64 (0.95-2.84)
Alcohol intake status			
Non-drinkers	194 (40.7)	189 (39.6)	1.00 (reference)
Drinkers	283 (59.3)	288 (60.4)	1.18 (0.71-1.76)

the analysis was carried out on the validated SNPs. The average call rate was 99.2%. Genotyping was carried out by Macrogen (Seoul, South Korea).

Statistical analysis

The study power was calculated using the "case-control for discrete traits" mode in the Genetic Power Calculator[22]. The following parameters were applied: risk allele frequency -0.4, alpha error -0.01, and disease prevalence -0.1%. The power of a codominant model was 0.7768 when the heterozygous OR was set to 1.5. For a dominant model, when the OR for a genotype with one or 2 risk allele(s) was taken as 2, the power was 0.8821. When a value of 2 was input for the OR for a genotype with 2 risk allele(s), the power of a recessive model was 0.8182.

Testing for deviation from the HWP was performed for each SNP in both cases and in controls using Pearson's χ^2 test. *D* values were measured using Lewontin's method for all combinations of biallelic loci[23,24], and linkage disequilibrium blocks were structured using Haploview version 4.2 (Daly Lab at the Broad Institute Cambridge, MA, United States). Haplotype blocks were constructed and statistically compared between cases and controls with SNP Analyzer version 2.0 (ISTEC Inc., Goyang, South Korea).

Student's *t* test was used to compare continuous variables between patients and control subjects. Associations between gastric cancer and the investigated SNPs and haplotypes were estimated *via* the OR and their corresponding 95%CI derived from multivariate conditional logistic regression models, after adjusting for potential confounding factors such as age, sex, smoking history, and alcohol intake. The genotypes of major homozygotes, heterozygotes and minor homozygotes were coded as 0, 1, and 2 in the codominant model, 0, 1 and 1 in the dominant model, and 0, 0 and 1 in the recessive model, respectively. Multiple testing corrections were carried out using Benjaminin and Hochberg's methods for controlling the false discovery rate (FDR)[25]. A two-sided adjusted *P* value of < 0.05 was considered statistically significant. FDR *Q* values were calculated separately for the SNPs and haplotypes based on these numbers. Gene-based association tests were performed using the versatile gene-based association study (VEGAS) method[26]. For these statistical analyses, SAS version 9.2 (SAS Institute, Cary, NC, United States) was employed.

RESULTS

Patient characteristics are summarized in Table 1. No significant difference was observed between the distributions of the age, sex, and smoking and drinking habits of the cases and controls.

Table 2 lists and provides the frequencies of the 15 selected SNPs in the study subjects. None of the polymorphisms were significantly deviated from Hardy-Weinberg equilibrium. All the minor allele frequencies of the cases and controls were greater than 10%.

The haplotype linkage disequilibrium blocks and haplotype frequencies for *ITGA1* are shown in Figure 1. *D* values were measured using Lewontin's method. Four block haplotypes were constructed using Haploview version 4.2. The common haplotypes (frequency > 10%) in each block accounted for 84.2%, 99.8%, 91.6% and 99.9% for the cases and 85.7%, 99.8%, 91.2% and 99.9% for the controls.

The observed associations between the genetic polymorphisms in the *ITGA1* gene and the risk of gastric cancer are shown in Table 3. In the codominant model, the OR of 1.517 obtained for SNP *rs2432143* (95%CI: 1.144-2.011; $P = 0.003$; FDR $Q = 0.045$) was statistically significant, even after controlling the FDR, and that for *rs2447867*, of 1.258 (95%CI: 1.051-1.505; $P = 0.012$; FDR $Q = 0.090$), was marginally significant. In the dominant model, the *rs1862610* and *rs2447867* polymorphisms were not statistically significant risk factors for gastric cancer, displaying ORs of 1.337 (95%CI: 1.029-1.737; $P = 0.029$; FDR $Q = 0.217$) and 1.412 (95%CI: 1.061-1.881; $P = 0.018$; FDR $Q = 0.217$), respectively. Only the *rs2432143* polymorphism was marginally significant in the recessive model, exhibiting an OR of 1.559 (95%CI: 1.150-2.114; $P = 0.004$; FDR $Q = 0.060$).

When the *P* values for the minor alleles of the codominant, dominant and recessive models were subjected to the VEGAS test, no significant gene-based associations were found. However, when the lower *P* value generated by the dominant and recessive models was input for every SNP, the value of the test statistic was 29.622, which was statistically significant ($P = 0.037$).

Four haplotype blocks were constructed using SNP Analyzer version 2.0. These blocks were evaluated for an association with the risk of gastric cancer (Table 4). The C-C type of *ITGA1* haplotype block 2 was marginally significant in the codominant model (OR = 0.602, 95%CI: 0.212-0.709; $P = 0.021$; FDR $Q = 0.063$) and was a significant protective factor against gastric cancer in the dominant model (OR = 0.653, 95%CI: 0483-0.884; $P = 0.006$; FDR $Q = 0.018$). In the dominant model, the A-T type of *ITGA1* haplotype block 2 was a significant risk factor (OR = 1.341, 95%CI: 1.034-1.741; $P = 0.027$; FDR $Q = 0.045$). No haplotype block was found to be significant in the recessive model.

Table 2 Frequency of *ITGA1* polymorphisms in cases and controls

SNP	Chromosomal position	Amino acid change	Genotype case/control				Case		Control	
							Frequency	HWE[1]	Frequency	HWE[1]
rs13188662	2686006	-	AA	AG	GG	N	0.280	0.573	0.276	0.597
			249/253	186/186	40/38	475/477				
rs11740785	2707341	-	AA	AC	CC	N	0.241	0.866	0.229	0.259
			279/290	166/156	32/31	477/477				
rs1820167	2713715	-	AA	AG	GG	N	0.435	0.806	0.420	0.904
			151/162	237/229	89/83	477/477				
rs1862610	2722239	-	CC	AC	AA	N	0.369	0.861	0.387	0.484
			172/205	223/192	82/80	477/477				
rs2432143	2725674	-	TT	TC	CC	N	0.104	0.671	0.146	0.658
			382/346	87/121	8/10	477/477				
rs2447867	2751733	C/C	CC	TC	TT	N	0.490	0.742	0.430	0.769
			123/155	241/229	113/89	477/473				
rs4865745	2770258	-	TT	TC	CC	N	0.270	0.892	0.268	0.124
			253/247	186/198	35/28	474/473				
rs13163497	2773367	-	GG	AG	AA	N	0.110	0.409	0.108	0.515
			375/381	97/89	4/7	476/477				
rs1904163	2780355	-	CC	TC	TT	N	0.298	0.196	0.272	0.698
			238/245	184/187	48/33	470/465				
rs1466445	2789486	-	CC	TC	TT	N	0.460	0.783	0.455	0.696
			139/142	233/229	101/100	473/471				
rs16880453	2789866	-	GG	GC	CC	N	0.466	0.914	0.465	0.424
			133/130	235/243	100/98	468/471				
rs2452864	2796757	-	TT	TC	CC	N	0.367	0.874	0.369	0.368
			190/183	224/230	63/59	477/472				
rs1275659	2828018	-	AA	AG	GG	N	0.257	0.185	0.278	0.864
			256/247	192/189	26/37	474/473				
rs1871186	2828974	-	TT	TC	CC	N	0.221	0.723	0.213	0.674
			287/296	166/157	22/23	475/476				
rs988574	2835169	E/G	TT	TC	CC	N	0.180	0.723	0.183	0.674
			319/309	141/155	15/9	475/473				

[1]*P* value for deviation from Hardy-Weinberg Equilibrium (HWE). SNP: Single nucleotide polymorphism.

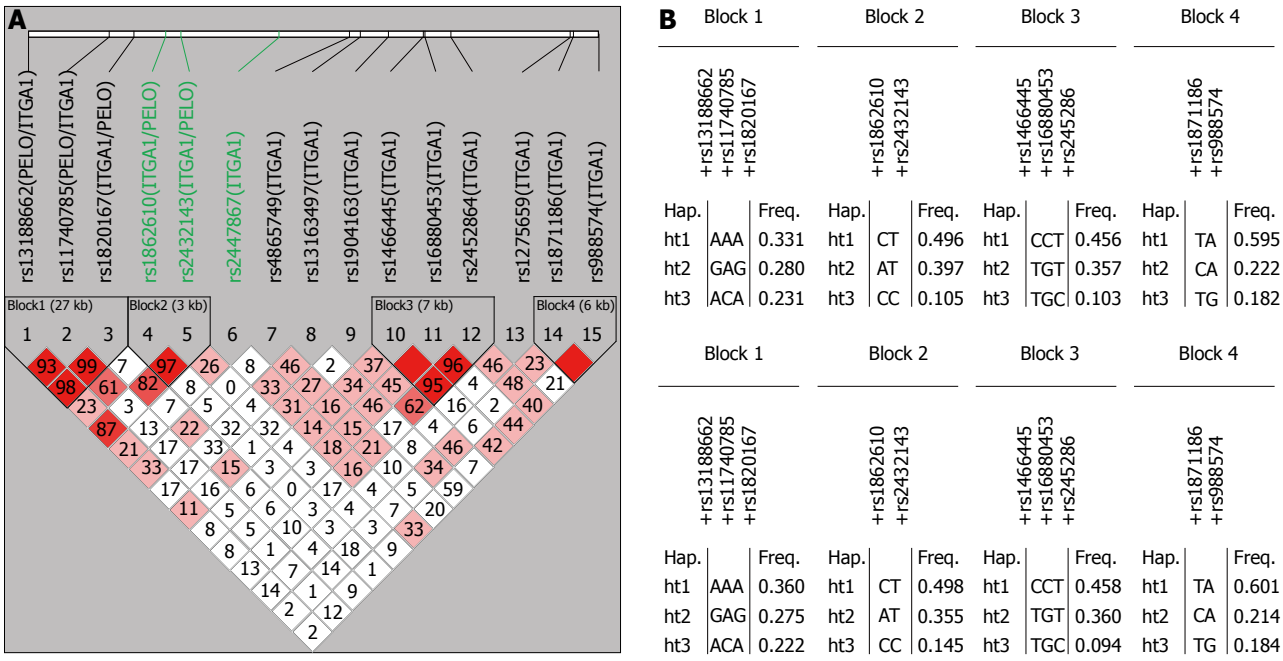

Figure 1 Haplotype linkage disequilibrium blocks and haplotype frequencies for *ITGA1*. A: Linkage disequilibrium (LD) blocks among *ITGA1* polymorphisms. Black squares indicate a statistically significant allelic association between a pair of single nucleotide polymorphisms, as measured by the *D* statistic; darker gray indicate higher values of *D*; B: Haplotype frequencies of *ITGA1* polymorphisms in cases (top) and controls (bottom).

Table 3 Association between *ITGA1* polymorphisms and gastric cancer in a case-control study of a Korean population

SNP	Chromosomal position	Codominant			Dominant			Recessive		
		OR (95%CI)	*P* value[1]	*Q*[2]	OR (95%CI)	*P* value[1]	*Q*[2]	OR (95%CI)	*P* value[1]	*Q*[2]
rs13188662	2686006	1.040 (0.840-1.281)	0.161	0.483	1.060 (0.811-1.379)	0.689	0.866	1.060 (0.660-1.690)	0.811	0.963
rs11740785	2707341	1.069 (0.869-1.313)	0.528	0.965	1.106 (0.848-1.442)	0.457	0.866	1.032 (0.630-1.692)	0.899	0.963
rs1820167	2713715	1.066 (0.884-1.286)	0.503	0.964	1.115 (0.846-1.468)	0.440	0.866	1.043 (0.751-1.447)	0.801	0.963
rs1862610	2722239	1.151 (0.965-1.372)	0.118	0.483	1.337 (1.029-1.737)	0.029	0.217	1.029 (0.740-1.429)	0.866	0.963
rs2432143	2725674	1.517 (1.144-2.011)	0.003	0.045	1.800 (0.603-5.371)	0.292	0.883	1.559 (1.150-2.114)	0.004	0.060
rs2447867	2751733	1.258 (1.051-1.505)	0.012	0.090	1.412 (1.061-1.881)	0.018	0.217	1.303 (0.966-1.756)	0.083	0.415
rs4865745	2770258	1.016 (0.829-1.246)	0.875	0.965	0.967 (0.750-1.247)	0.795	0.863	1.269 (0.759-2.122)	0.363	0.927
rs13163497	2773367	1.021 (0.768-1.357)	0.884	0.965	1.064 (0.781-1.449)	0.693	0.866	0.571 (0.167-1.952)	0.371	0.927
rs1904163	2780355	1.157 (0.943-1.420)	0.161	0.483	1.104 (0.849-1.436)	0.461	0.866	1.593 (0.984-2.577)	0.058	0.415
rs1466445	2789486	1.013 (0.845-1.213)	0.890	0.965	1.032 (0.778-1.368)	0.829	0.883	1.000 (0.736-1.358)	1.000	1.000
rs16880453	2789866	1.000 (0.832-1.201)	1.000	1.000	0.979 (0.734-1.305)	0.883	0.883	1.025 (0.752-1.398)	0.874	9.632
rs2452864	2796757	0.986 (0.816-1.191)	0.885	0.965	0.947 (0.728-1.233)	0.687	0.883	1.056 (0.728-1.532)	0.775	9.632
rs1275659	2828018	1.136 (0.919-1.404)	0.237	0.592	1.522 (0.899-2.575)	0.117	0.585	1.095 (0.841-1.427)	0.500	9.632
rs1871186	2828974	1.043 (0.841-1.293)	0.701	0.965	1.072 (0.828-1.388)	0.597	0.866	0.957 (0.533-1.716)	0.881	9.632
rs988574	2835169	0.985 (0.772-1.256)	0.901	0.965	0.927 (0.707-1.215)	0.581	0.866	1.667 (0.729-3.808)	0.225	0.843
VEGAS statistics (*P*)		23.986 (0.105)			16.823 (0.364)			18.732 (0.260)		

[1]*P* values for logistic analysis of three alternative models (codominant, dominant and recessive); [2]False discovery rate *Q* value. When the lower *P* value generated by the dominant and recessive models was applied for every single nucleotide polymorphism (SNP), the value of the versatile gene-based association study (VEGAS) statistic was 29.622 (*P* = 0.037).

Table 4 Association between *ITGA1* haplotypes and gastric cancer

Haplotypes		Codominant			Dominant			Recessive		
		OR (95%CI)	*P* value[1]	*Q*[2]	OR (95%CI)	*P* value[1]	*Q*[2]	OR (95%CI)	*P* value[1]	*Q*[2]
ITGA1	AAA	0.771 (0.510-1.165)	0.414	0.973	0.860 (0.666-1.112)	0.250	0.750	0.819 (0.555-1.210)	0.316	0.913
Haplotype	GAG	1.039 (0.643-1.678)	0.973	0.973	1.030 (0.799-1.328)	0.819	0.819	1.026 (0.644-1.636)	0.913	0.913
block 1	ACA	0.992 (0.559-1.760)	0.768	0.973	1.088 (0.839-1.410)	0.525	0.787	0.957 (0.544-1.683)	0.879	0.913
ITGA1	CT	0.982 (0.688-1.407)	0.640	0.640	1.072 (0.800-1.437)	0.641	0.641	0.911 (0.679-1.223)	0.536	0.536
Haplotype	AT	1.316 (0.686-1.407)	0.086	0.129	1.341 (1.034-1.741)	0.027	0.045	1.121 (0.784-1.603)	0.532	0.536
block 2	CC	0.602 (0.212-0.709)	0.021	0.063	0.653 (0.483-0.884)	0.006	0.018	0.661 (0.233-1.872)	0.433	0.536
ITGA1	CCT	1.023 (0.707-1.480)	0.677	0.794	0.934 (0.705-1.236)	0.631	0.916	0.819 (0.555-1.210)	0.316	0.913
Haplotype	TGC	0.973 (0.641-1.475)	0.314	0.794	0.986 (0.761-1.278)	0.916	0.916	1.026 (0.644-1.636)	0.913	0.913
block 3	TGT	1.418 (0.446-4.507)	0.794	0.794	1.084 (0.782-1.505)	0.627	0.916	0.957 (0.544-1.683)	0.879	0.913
ITGA1	TA	0.938 (0.641-1.370)	0.907	0.907	0.928 (0.658-1.310)	0.671	0.671	0.997 (0.765-1.299)	0.981	0.981
Haplotype	CA	0.983 (0.536-1.803)	0.803	0.907	1.079 (0.832-1.400)	0.567	0.671	0.952 (0.523-1.733)	0.873	0.981
block 4	TG	1.619 (0.698-3.756)	0.320	0.907	0.925 (0.708-1.209)	0.569	0.671	1.685 (0.730-3.888)	0.217	0.981

[1]*P* values for logistic analysis of three alternative models (codominant, dominant and recessive). The *P* value for haplotype associations were calculated using single nucleotide polymorphisms Analyzer™ 2.0 software; [2]False discovery rate *Q* value.

DISCUSSION

The present study focused on the association of genetic polymorphisms and haplotypes of the *ITGA1* gene with gastric cancer risk. It has been suggested that the integrin α1 subunit could be involved in gastric cancer carcinogenesis. Integrins on gastric epithelial cells have been reported to serve as a portal for the entry of *H. pylori cagA*[11]. Additionally, the integrin α1 subunit is involved in the adhesion and dissemination of gastric cancer cells to the peritoneum[18], and an *ITGA2* polymorphism has been reported to be associated with an increase in the risk of gastric cancer[20]. However, to our knowledge, no previous study has examined the association between *ITGA1* polymorphisms and the risk of gastric cancer.

The SNPs *rs1862610*, *rs2432143* and *rs2447867* were significantly associated with an increase in the risk of gastric cancer. After controlling the FDR, only SNP *rs2432143* in the codominant model was statistically significant. In a gene-based association test, the *ITGA1* gene was found to be significantly associated with gastric cancer.

The C-C type of *ITGA1* haplotype block 2, which includes *rs1862610* and *rs2432143* in intron 1 of the *ITGA1* gene, was found to be a significant protective factor and the A-T type to be a risk factor for gastric cancer. This statistical significance was maintained after controlling the FDR. However, the precise molecular mechanism related to these SNPs is not clear. Based on SNP function prediction using computational methods, SNPs *rs1862610* and *rs2432143* are not predicted to be involved in any structural or functional changes in the integrin α1 subunit. However, we cannot rule out the possibility that these SNPs are either associated with the stability of *ITGA1* mRNA, or in linkage disequilibrium with an as yet unknown functional polymorphism affecting the ex-

pression or function of the integrin α1 subunit.

We used public databases of SNPs related to gastric cancer and assessed the potential functions of selected SNPs with SNP function prediction software. Among the 15 selected SNPs, only two were located in exons, and one was non-synonymous. The potential function was not predicted for any of these SNPs, except for *rs2447867*, which was predicted to be an exonic splicing enhancer (ESE). ESEs are clinically significant because synonymous point mutations in ESEs that were previously thought to be silent mutations can lead to exon skipping and the production of a non-functional protein. As loss of integrin α1β1 has been observed in some other malignancies[27], non-functional integrin α1β1 could be associated with gastric cancer.

The increased expression of integrin molecules by epithelial cells during inflammation of the underlying lamina propria is probably an adaptive response to prevent extensive epithelial cell sloughing caused by inflammatory mediators. Loss of epithelial integrity due to a decrease in the function of integrin results in more severe injury of the epithelium[21]. At these sites of tissue injury, bone marrow-derived cells are recruited, and these cells can be a potential source of malignancy[28]. Because chronic infection with *H. pylori* also induces repopulation of the stomach with bone marrow-derived cells, there is a possibility that a non-functional integrin α1 subunit and *H. pylori* infection would have a synergistic effect in increasing the risk of gastric cancer. The major limitation of the present study is that we did not test for the presence of antibodies against *H. pylori* and the *cagA* antigen in the sera of the case and control subjects.

The OR obtained for SNPs *rs1862610*, *rs2432143*, and *rs2447867* were all below 1.6, while the OR for the *ITGA2* C807T polymorphism in relation to gastric cancer in a Chinese population is 1.57[20]. These relatively small values can be explained by the promiscuity and redundancy of integrins: one integrin can bind several different ligands, and many different integrins can bind to the same ligand[29]. Therefore, if an integrin is not functioning, other integrins can compensate for at least some of its function.

In conclusion, the *ITGA1* gene SNPs *rs2432143* and *rs2447867* and the *ITGA1* haplotype block that includes SNP *rs2432143* are significantly associated with gastric cancer risk.

COMMENTS

Background

Integrins mediate signaling events that are essential for stable cell adhesion, cell spreading, migration, survival, proliferation and differentiation. Several integrins, including α1β1, bind to extracellular matrix proteins present in the basal membranes of mature vessels. Tumor progression and the metastasis of various cancers are associated with integrins. The *ITGA1* gene, located on chromosome 5q11.2, encodes the integrin α1 subunit, which is involved in the adhesion of gastric cancer cells to the peritoneum. Adhesion of integrin α1-positive gastric cancer cells to the extracellular matrix is a critical process in peritoneal dissemination. As integrin α1β1 is up-regulated during inflammation in the gastrointestinal tract mucosa, which is the first step in gastric carcinogenesis, it is possible that the integrin α1 subunit plays an important role in the development of gastric cancer. It has been suggested that the integrin α1 subunit could be involved in gastric cancer carcinogenesis. Integrins on gastric epithelial cells have been reported to serve as a portal for the entry of *Helicobacter pylori* (*H. pylori*) *cagA*. As integrin α1β1 is up-regulated during inflammation in the gastrointestinal tract mucosa, which is the first step in the gastric carcinogenesis, it is possible that the integrin α1 subunit plays an important role in the development of gastric cancer.

Research frontiers

There are few studies addressing the role of integrins in the development of gastric cancer. An association with an increased risk of gastric cancer has only been reported previously for the *ITGA2* C807T polymorphism in a Chinese population. No earlier study has focused on the association of *ITGA1* gene single nucleotide polymorphisms (SNPs) and haplotypes with gastric cancer risk.

Innovations and breakthroughs

To the best of the authors' knowledge, this present study is the first to suggest a significant association of the genetic polymorphisms and haplotypes of *ITGA1* gene with an increased gastric cancer risk.

Applications

Integrins on gastric epithelial cells have been reported to serve as a portal of entry for *H. pylori cagA*, and loss of epithelial integrity due to a decrease in the function of integrins results in more severe injury of the epithelium. Studies are needed addressing the interaction of non-functional integrin α1 subunit and *H. pylori* infection in increasing the risk of gastric cancer.

Peer review

This paper is focused on the *ITGA1* polymorphisms and haplotypes, and gastric cancer risk in a Korean population. The results showed the SNPs *rs1862610*, *rs2432143*, and *rs2447867*, and the *ITGA1* haplotype block which includes SNPs *rs1862610* and *rs2432143* were significantly associated with gastric cancer. It is interesting.

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BRIEF ARTICLE

Non-invasive assessment of choledocholithiasis in patients with gallstones and abnormal liver function

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Abstract

AIM: To find a non-invasive strategy for detecting choledocholithiasis before cholecystectomy, with an acceptable negative rate of endoscopic retrograde cholangiopancreatography.

METHODS: All patients with symptomatic gallstones were included in the study. Patients with abnormal liver functions and common bile duct abnormalities on ultrasound were referred for endoscopic retrograde cholangiopancreatography. Patients with normal ultrasound were referred to magnetic resonance cholangiopancreatography. All those who had a negative magnetic resonance or endoscopic retrograde cholangiopancreatography underwent laparoscopic cholecystectomy with intraoperative cholangiography.

RESULTS: Seventy-eight point five percent of patients had laparoscopic cholecystectomy directly with no further investigations. Twenty-one point five percent had abnormal liver function tests, of which 52.8% had normal ultrasound results. This strategy avoided unnecessary magnetic resonance cholangiopancreatography in 47.2% of patients with abnormal liver function tests with a negative endoscopic retrograde cholangiopancreatography rate of 10%. It also avoided un-necessary endoscopic retrograde cholangiopancreatography in 35.2% of patients with abnormal liver function.

CONCLUSION: This strategy reduces the cost of the routine use of magnetic resonance cholangiopancreatography, in the diagnosis and treatment of common bile duct stones before laparoscopic cholecystectomy.

Key words: Magnetic resonance cholangiopancreatography; Endoscopic retrograde cholangiopancreatography; Choledocholithiasis; Liver function tests; Laparoscopic cholecystectomy; Obstructive jaundice

Core tip: This strategy reduces the cost of the routine use of magnetic resonance cholangiopancreatography, in the diagnosis and treatment of common bile duct stones before laparoscopic cholecystectomy.

Al-Jiffry BO, Elfateh A, Chundrigar T, Othman B, AlMalki O, Rayza F, Niyaz H, Elmakhzangy H, Hatem M. Non-invasive assessment of choledocholithiasis in patients with gallstones

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INTRODUCTION

Gallstone disease is one of the most common surgical problems worldwide. Both environmental and genetic factors are known to contribute to susceptibility to the disease[1]. It has been reported that in Saudi Arabia there is an increasing incidence of gallstone disease[2], especially in the high altitude provinces in the Asir[3] and Taif regions[4], and similar findings have been reported for other countries that have similar environmental and nutritional factors[5,6]. Complications of gallstone disease vary from simple recurrent biliary colic to life-threatening conditions such as ascending cholangitis and pancreatitis. In addition, the disease is thought to be a risk factor for developing pancreaticobiliary cancer, particularly in patients with choledocholithiasis [common bile duct stones (CBDS)], approximately 10% of patients with symptomatic gallstones[7].

Since symptomatic gallstone is a common indication for surgery, an accurate preoperative detection of CBDS is imperative in order to decrease operative risks and health care costs[7-9]. Better detection and treatment of CBDS before laparoscopic cholecystectomy (LC) would help deliver an appropriate, fast, and cost effective treatment[10]. Liver function tests (LFTs) and abdominal ultrasonography (USG), combined with medical history and clinical examinations are currently the standard preoperative methods used to diagnose patients with gallstones[9-14]. However, this approach is often not accurate enough to establish a firm diagnosis of CBDS[4,8,9].

Imaging tests are routinely used to confirm a choledocholithiasis diagnosis. While abdominal USG is the most commonly used screening modality, other imaging tests used for this purpose include endoscopic and laparoscopic USG, magnetic resonance cholangiopancreatography (MRCP), intraoperative cholangiography (IOC) and endoscopic retrograde cholangiopancreatography (ERCP).

ERCP was the gold standard for diagnosing and treating CBDS; however, it is invasive, has associated morbidity and mortality, and has been shown to have a negative rate up to 75% in some studies[5]. Therefore, it was abandoned as a diagnosing method and used only for stone extraction.

MRCP is a noninvasive procedure with no associated morbidity that has become the gold standard in diagnosing CBDS; however, it should only be used when proper indications are observed[9-14] due to its high cost and limited availability[11,15,16]. Many authors have proposed its routine use[17-19] in all patients with suspected CBDS, however, in high probability patients, its cost and the need for ERCP to remove the stones makes it questionable.

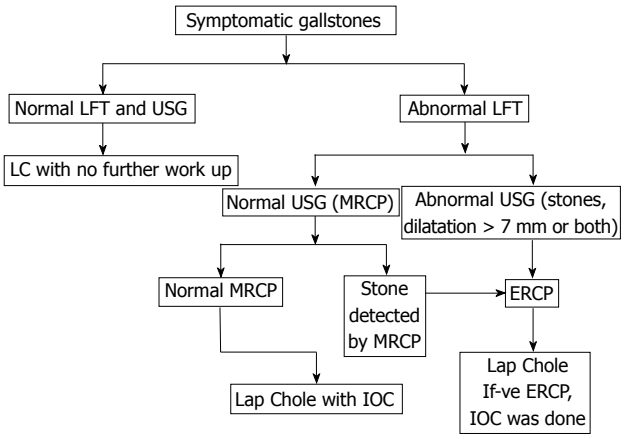

Figure 1 Algorithm followed in the management of symptomatic gallstones. LFT: Liver function test; USG: Ultrasonography; LC: laparoscopic cholecystectomy; MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography; IOC: Intraoperative cholangiography.

IOC is particularly valuable in patients with unclear anatomy and those with preoperative predictors of choledocholithiasis but negative findings on MRCP[20,21]. Many scoring indices and guidelines have been developed to determine acceptable preoperative indications for IOC, but none so far have proved satisfactory[9,22-24]. Therefore, the aim of the current study was to find a non-invasive preoperative approach, using MRCP, ERCP, and IOC, to diagnose and treat CBDS prior to performing LC.

MATERIALS AND METHODS

We conducted a prospective study on all patients with symptomatic gallstones who presented at Al Hada Armed Forces Hospital in Taif, Saudi Arabia from April 2006 to April 2010. Patients not fit for surgery were excluded from the study. In addition, patients who presented with acute pancreatitis were excluded since we have a different protocol for them in our center. The study was approved by the local committee on human research, and all patients gave written informed consent. All patients underwent detailed preoperative evaluations consisting of clinical history, laboratory testing including LFT, (serum bilirubin, alkaline phosphatase, serum glutamic-oxaloacetic transaminase and serum glutamic pyruvic transaminase), and USG examination.

An algorithm was designed (Figure 1). Patients with normal LFT and no bile duct abnormalities were referred for LC without further work-up. Patients with abnormal LFT and USG proven CBDS and/or bile duct dilatation > 7 mm were referred for ERCP for stone removal, followed by LC. Patients with abnormal LFT and normal bile ducts (determined by USG) were referred for MRCP, and if CBDS were detected, they were referred for ERCP for stone removal, followed by LC. MRCP and ERCP negative cases underwent LC with IOC to detect false negative cases and avoid retained stones. For these patients, intraoperative discovery of CBDS would indicate

Table 1 Demographic data *n* (%)

Characteristic	Value
Total number of patients	896
Female patients	717 (80)
Male patients	179 (20)
Mean age of females (yr)	41.4 ± 21.3
Mean age of males (yr)	45.0 ± 21.6
Number of patients underwent LC without MRCP or/and ERCP	703 (78.5)
Number of patients with abnormal liver functions.	193 (21.5)
Female patients	116 (60)
Male patients	77 (40)
Mean age of females (yr)	55.6 ± 19.6
Mean age of males (yr)	60.7 ± 19.8

ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography; LC: Laparoscopic cholecystectomy.

Table 2 Radiological findings in patients with abnormal liver function tests *n* (%)

Findings	Patient	Stones removed by ERCP	Mean T.Bili (mg/dL)	Mean Al-P (ratio to normal)
Total	193	109	3.7	1.7
Abnormal CBD on USG (percent of total)	91 (47.2)	82 (90)	4.2	2
CBD dilatation	28 (30.8)	24 (85.7)	4.5	2
CBD stones	23 (25.3)	20 (87)	4.3	2.2
Both	40 (43.9)	38 (90)	4	1.8
Normal CBD on USG, MRCP findings (percent of total)	102 (52.8)	27 (26.5)	3.3	1.4
Normal MRCP	70 (68.6)	2 (2.9)	3.2	1.3
Stones on MRCP	32 (31.4)	25 (78.2)	3.4	1.7

MRCP: Magnetic resonance cholangiopancreatography; Al-P: Alkaline phosphatase; T.Bili: Total bilirubin; USG: Ultrasonography; CBD: Common bile duct; LFT: Liver function test; ERCP: Endoscopic retrograde cholangiopancreatography.

stone removal by ERCP in the same sitting with the LC.

Statistical analysis

SPSS 18.0 (SPSS, Chicago Illinois) was used for carrying out statistical analysis. Group differences were further analyzed by χ^2 and difference between means of continuous variables was tested by Student's *t* test. Multivariate logistic regression analysis was adopted to control for confounders and level of significance was determined at $P < 0.05$.

RESULTS

A total of 896 patients were included in the study. Table 1 shows the patient demographic information. Out of these, 703 (78.5%) patients underwent LC without any further workup. Patients who had abnormal LFTs [193 (21.5%)] were older and there were more males in this group.

Table 2 demonstrates the breakdown of all the patients with abnormal LFT. CBD abnormalities were detected on USG in 91 (47.2%) patients and ERCP was used to extract stones in 90% of them. Abnormal LFT results, in which USG found dilatation but no stones were observed in 28 patients. The mean CBD diameter was 8.8 mm in these patients, with stones being extracted by ERCP in 85.7%. In 40 patients with abnormal LFT results and for whom USG detected both dilatation and stones, the mean CBD diameter was 9 mm, with stones being extracted by ERCP in 90%.

Normal bile ducts were detected by USG in 102 (52.8%) patients and ERCP was used to extract stones in 26.5% of them. IOC detected two patients with CBDS in the MRCP negative group (false negative 2.9%) and none in the ERCP negative group. There were seven patients with false positive MRCP where the ERCP did not detect any stones (false positive of 21%). This high percentage could be explained by the time between the MRCP and the ERCP that is about 2-3 d because of which the stones could have passed.

More importantly when looking at this group, 29/102 (28.4%) patients had a total bilirubin > 4 mg/dL which is counted as a high risk in recent guide lines (9); out these 17 (58.6%) patients did not have stones on IOC and ERCP was not conducted in this group of patients.

Stones were confirmed in 90% of the patients with an abnormal LFT and USG findings. ERCP detected stones in 24.5% of the patients with normal findings. This strategy avoided the use of MRCP in 47.2% of patients with abnormal LFT with a negative rate for the ERCP of 10% only. It also helped avoid unnecessary ERCP in 17 (58.6%) patients with total bilirubin > 4 mg/dL.

Statistical findings are shown in Table 3, where patients with abnormal USG, total bilirubin and alkaline phosphatase had a significant stone prediction in a univariate analysis. After controlling for confounders in multivariate Logistic regression analysis Alkaline phosphatase and USG findings were found to be significant predictors for CBDS. However, total bilirubin was found not to be a significant predictor (Table 4).

Multivariate statistical analysis found that the rise in alkaline phosphatase was a significant predictor for CBDS. It became highly significant when double the normal alkaline phosphatase value. In this case, the probability of stone detection by ERCP increased 30-fold when

Table 3 Final endoscopic retrograde cholangiopancreatography findings

Presence of stones	Patient	US abnormal	Mean T.Bili (mg/dL)	Mean Al-P (ratio to normal)
Stones	109 (56.5)	82 (90)	4.3 ± 2.1	1.9 ± 0.8
No stones	84 (43.5)	9 (10)	2.9 ± 1.3	1.3 ± 0.6
P value		< 0.0001	< 0.01	< 0.001
Total	193	91	3.7	1.7

Data are expressed as absolute numbers (percentage) or mean ± SD. MRCP: Magnetic resonance cholangiopancreatography; Al-P: Alkaline phosphatase; T.Bili: Total bilirubin; US: Ultrasound.

Table 4 Multivariate analysis *n* (%)

Findings	Common bile duct stones		OR[1]	95%CI[1]
	Present	Absent		
Alkaline phosphatase				
< 151 U	10 (23.3)	33 (76.7)	1	
151-225 U	37 (54.4)	31 (45.6)	3.1	7.00-9.88
226-300 U	21 (58.0)	15 (42.0)	4.5	1.23-16.49
> 300 U	41 (89.0)	5 (11.0)	29.8	7.28-121.54
Ultrasound findings				
Normal	27 (26.5)	75 (73.5)	1	
Dilatation	24 (86.0)	4 (14.0)	19.8	5.41-72.42
Stones	20 (87.0)	3 (13.0)	14.3	3.53-58.17
Both	38 (95.0)	2 (5.0)	61	13.03-285.1

[1]The adjusted measure of association between risk factors and common bile duct stones was expressed as the OR with 95%CI. Adjusted or crude ORs with 95%CI that did not include 1.0 were considered significant.

this enzyme level was within the normal range.

In addition, in USG findings that detected CBD dilatation and those that detected stones were both significant predictors for the presence of CBDS found by ERCP. Detection of both dilation and stones concurrently was a highly significant predictor of the presence of CBDS, which were about 60 times more likely than when USG results were normal.

DISCUSSION

CBDS can remain hidden for years, frequently passing undetected into the duodenum. When the symptoms become apparent, the presentation will likely include obstructive jaundice or more serious complications such as acute pancreatitis or cholangitis[7,25]. Preoperative detection and intervention to remove these stones is vital[8]. An increase in bilirubin and alkaline phosphatase levels may be the only evidence of choledocholithiasis[10-12]. USG examination may confirm the presence of CBDS but cannot definitively exclude them when not detected[10]. The gold standard for treating these stones is ERCP, which has sensitivity and specificity rates both around 95%[8,11,14,26,27]. However, if the clinical, radiological, and laboratory testing indicates a low probability of choledocholithiasis, less invasive method such as MRCP should be performed first[9].

The sensitivity of transabdominal USG is low for detecting CBDS (22%-55%), but it is better at detecting CBD dilatation (sensitivity 77%-87%). For patients with abnormal LFT which USG detects CBDS, successful diagnosis of choledocholithiasis has been reported to be above 80%. Negative CBDS detection by ERCP in such patients may be related to the passage of these stones into the duodenum before the procedure[4,28,29].

The diameter of a normal bile duct is 3-6 mm. it increases by one mm every 10 years after the age of 60, causing mild dilatation in the elderly[30]. CBD greater than 8 mm in patients with an intact gallbladder is usually indicative of biliary obstruction[9]. Although no single factor strongly predicts choledocholithiasis in patients with symptomatic gallstones, many studies have shown that the probability of CBDS is higher in the presence of multiple abnormal prognostic signs. Patients with such markers are considered to be at high risk, and preoperative ERCP is indicated when there are no available facilities for performing CBD laparoscopic exploration[9-13,31-34].

In the present study, abnormal LFT results were observed in 21.5% in patients with symptomatic gallstones, a higher incidence than previously reported in the literature (15%)[35]. This discrepancy may be related to environmental (Taif is a high-altitude province), cultural or social factors[2-4,6,7]. Our facility is a tertiary hospital serving a widespread rural area.

Therefore, the routine use of MRCP as has been recommended by others for patients with abnormal LFT[17-19] is not practical or cost effective.

Among the patients involved in the study, there were 78.5% patients with normal LFT results and no CBD abnormalities detected by USG. They were therefore referred for LC without further workup, consistent with the recommendations made by the Standards of Practice Committee of the American Society for Gastrointestinal Endoscopy (SPC-ASGE)[9]. The results of our study, which concluded before the publication of the 2010 guidelines, were similar, patients with symptomatic gallstones but normal LFT and USG are considered to be at low risk for choledocholithiasis. For these patients, they recommend, as we do, cholecystectomy, without further evaluation to avoid the cost and risks of additional preoperative biliary evaluation, which are not justified by the low probability of CBDS[9]. Whether or not to perform routine IOC or laparoscopic US during LC remains an area of controversy[20,21].

We found (52.8%) patients with abnormal LFT but normal CBD USG results, who were sent for MRCP examination. This group of patients is considered by the SPC-ASGE to be at intermediate risk of choledocholithiasis, and their guidelines recommend further evaluation with preoperative EUS or MRC or an IOC[9], as we do. However, they do recommend that a total bilirubin higher than 4 mg/dL, should be considered as a high probability of CBDS. In our study, we found total bilirubin to be not a significant predictor on multivariant analysis. Also, in 17 (58.6%) of the patients with high total bilirubin (> 4 mg/dL) and normal USG, CBDS was not detected by IOC in the operating room. Therefore, as per their recommendation ERCP was avoided in this group in our study.

Statistical findings revealed that a rise in bilirubin level was not a significant predictor for detecting CBDS. However, this finding should be reevaluated considering the higher incidences of hepatitis, sickle cell anemia, and secondary polycythemia (related to the high altitude) in our province. Yang *et al*[36] found that among the components of the LFTs, alkaline phosphatase was a better indicator for choledocholithiasis than bilirubin. However, the SPC-ASGE reported modestly better CBDS positive predictive values for bilirubin. They found cholestatic

liver biochemical tests, in particular alkaline phosphatase and γ-glutamyl transpeptidase, increases progressively with the duration and severity of biliary obstruction.

In the present study, MRCP helped avoid unnecessary ERCP in 58.6% of patients with high total bilirubin and normal CBD USG results. It was associated with a false negative rate of 2.96% and false positive rate of 21%, similar to those reported in other studies[16,37].

Patients with abnormal LFT and USG were classified as a high risk for CBDS. By applying this algorithm, we diagnosed and treated these patients directly with ERCP and avoided the cost of MRCP and stones were extracted in 90%, with a low negative incidence of ERCP of 10%. This led to a shorter hospital stay and was even far better when some patients had the ERCP and the LC in the same sitting.

IOC has multiple advantages, as some centers use it routinely to identify the biliary anatomy and others use it for stone detection[15,16]. Its routine use is still controversial; however, in selective cases it is widely adopted. In cases where CBDS are thought of, it can be used with less cost and in the same time as the LC where it will take few minutes. However, not many general surgeons are familiar with this use, making it a less popular procedure. Therefore, its use in selective cases has been accepted. We have recommended its use in patients with negative MRCP or ERCP since these procedures have a false negative rate and discharging these patients with retained CBDS can lead to delayed acute presentation like acute pancreatitis, cholangitis and cystic duct leak[25,38].

In conclusion, we recommend the use of this simple algorithm to stratify patients into low risk when LFT and USG are normal. These patients can go for LC with no further work-up. Patients with abnormal LFT or US are stratified as intermediate risk regardless of the total bilirubin level and should undergo MRCP as the risk of ERCP is not justified. Patients with abnormal LFT and USG are stratified as high risk and should undergo ERCP and stone extraction with LC in the same sitting if possible, as the cost of MRCP and the time needed is not justified.

COMMENTS

Background

Symptomatic gallstones with abnormal liver function tests are seen in higher percentages in higher altitude areas. Choledocholithiasis is the commonest cause; however, it is a challenge to diagnose and treat these cases while maintaining a low cost and minimum hospital stay. In this study the authors tried to design a simple pathway to diagnose and treat this problem without the over use of magnetic resonance cholangiopancreatagrophy that is costly or the use of endoscopic retrograde cholangiography that has major side effects.

Research frontiers

In the past all patients with common bile duct stones (CBDS) were subjected to endoscopic retrograde cholangiopancreatography (ERCP). This was the best way to diagnose these patients, however, there were complications like bleeding, perforation or even death with this procedure. Then magnetic resonance cholangiopancreatography (MRCP) was developed and became widely used. However, it is very costly and after the diagnosis of CBDS an ERCP was needed to remove the stone. Recently some authors have advocated the routine use of MRCP without looking in the cost or even its availability. The authors believe that it is time to reach a balance between the uses of both procedures to get the best of both when needed.

Innovations and breakthroughs

The authors have developed a simple pathway for the treatment of CBDS which has the least cost and requiring a minimal hospital stay. The authors have also found that the total bilirubin level does not play a part in the pathway as mentioned by the American Endoscopy Society.

Applications

This simple pathway can be applied in any patient care facility even if it is not very advanced. The pathway does not have any special requirements.

Peer review

The manuscript is quite well written. The methods are adequate. The results justify the conclusions drawn.

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BRIEF ARTICLE

Erosive esophagitis associated with metabolic syndrome, impaired liver function, and dyslipidemia

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Abstract

AIM: To investigate whether erosive esophagitis is correlated with metabolic syndrome and its components, abnormal liver function, and lipoprotein profiles.

METHODS: We conducted a cross-sectional, case control study of subjects who underwent upper endoscopy during a health examination at the Health Management and Evaluation Center of a tertiary medical care facility located in Southern Taiwan. Metabolic syndrome components, body mass index (BMI), liver function, dyslipidemia, and cardiovascular risk factors, as defined by the ratio of total cholesterol to high-density lipoprotein cholesterol (HDL-C), and the ratio of low-density lipoprotein cholesterol to HDL-C were compared between individuals with and without erosive esophagitis. Risk factors for erosive esophagitis were evaluated by multivariate logistic regression.

RESULTS: Erosive esophagitis was diagnosed in 507 of 5015 subjects who were individually age and sex matched to 507 esophagitis-free control subjects. In patients with erosive esophagitis, BMI, waist circumference, blood pressure, fasting plasma glucose, triglyceride levels, aspartate aminotransferase, alanine aminotransferase, the ratio of total cholesterol to HDL-C, and the ratio of low-density lipoprotein cholesterol to HDL-C were significantly higher and HDL-C was significantly lower compared to patients without erosive esophagitis (all $P < 0.05$). In a multivariate analysis, central obesity (OR = 1.38; 95%CI: 1.0-1.86), hypertension (OR = 1.35; 95%CI: 1.04-1.76), hypertriglyceridemia (OR = 1.34; 95%CI: 1.02-1.76), cardiovascular risk factors as defined by a ratio of total cholesterol to HDL-C > 5 (OR = 1.45; 95%CI: 1.06-1.97), and aspartate aminotransferase (OR = 1.59; 95%CI: 1.08-2.34) were significantly associated with erosive esophagitis.

CONCLUSION: Metabolic syndrome, impaired liver function, and a higher ratio of total cholesterol to HDL-C were associated with erosive esophagitis.

Key words: Erosive esophagitis; Metabolic syndrome; Central obesity; Abnormal liver function; Dyslipidemia

Core tip: A cross-sectional, case control study of subjects who underwent upper endoscopy during a health examination was conducted. Metabolic syndrome components, body mass index, liver function, and dyslipidemia were compared between individuals with and without erosive esophagitis. Risk factors for erosive esophagitis were evaluated. Erosive esophagitis was diagnosed in 507 of 5015 subjects who were individually age- and sex-matched to 507 esophagitis-free control

subjects. In addition to metabolic syndrome, we also found that abnormal liver function and predictors of future coronary heart disease were associated with erosive esophagitis.

Loke SS, Yang KD, Chen KD, Chen JF. Erosive esophagitis associated with metabolic syndrome, impaired liver function, and dyslipidemia. *World J Gastroenterol* 2013; 19(35): 5883-5888 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5883.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5883

INTRODUCTION

The prevalence of erosive esophagitis in Asian countries has dramatically increased during the last two decades[1-3]. The prevalence of erosive esophagitis in the Taiwanese adult population is estimated to be 10%-15%[1,4]. Although the mechanism underlying this increase in prevalence remains to be determined, several risk factors of erosive esophagitis have been identified, including male sex, hiatal hernia, smoking, alcohol consumption, and obesity[5-7]. Metabolic syndrome is a complex disorder comprising central obesity, high blood pressure (BP), hyperglycemia, hypertriglyceridemia, and a low concentration of high-density lipoprotein cholesterol (HDL-C). In addition to being associated with cardiovascular disease and diabetes mellitus, metabolic syndrome and its component elements have also been associated with various gastrointestinal diseases and abnormal liver function[7-12]. The correlation between erosive esophagitis and body mass index (BMI) is controversial[13,14], as is the association between erosive esophagitis and hypertriglyceridemia or hyperglycemia[5,13-15]. This study was undertaken to characterize the correlation between erosive esophagitis and metabolic syndrome and its components, abnormal liver function and abnormal lipoprotein profiles - that have been used to predict coronary heart disease.

MATERIALS AND METHODS

Subjects

This study was designed as a cross-sectional, case control study. From January 2008 to December 2008, 5981 subjects visited the Health Management and Evaluation Center of a tertiary medical care facility located in Southern Taiwan for routine health examinations. Our center offers a variety of healthcare tests and procedures, including upper gastrointestinal endoscopy. The majority of subjects underwent a self-paid physical check-up; others were employees coming for their regular medical check-up. Most of the subjects were free of symptoms and were not chronic alcohol drinkers. Of the 5031 subjects who underwent upper gastrointestinal endoscopy, 507 were diagnosed with erosive esophagitis. The severity of erosive esophagitis was graded from A-D according to the Los Angles classification[16]. We matched each case subject, according to age and gender, with one control selected from the 4508 subjects with normal upper endoscopic findings. The study was approved by the institutional review board of the hospital in which the study was conducted.

Definition of metabolic syndrome and obesity

In this study, metabolic syndrome was defined according to the modified National Cholesterol Education Program Adult Treatment Panel Ⅲ for Asian populations. The waist circumference cutoff measurement was altered according to the criteria of the Bureau of Health Promotion, Department of Health, because the absolute risk of diabetes and cardiovascular disease is greater in Asians with a lesser degree of obesity[17,18]. Metabolic syndrome was diagnosed when at least three of the following criteria were found: (1) waist circumference $\geqslant$ 90 cm for men and $\geqslant$ 80 cm for women; (2) systolic BP $\geqslant$ 130 mmHg, diastolic BP $\geqslant$ 85 mmHg, or current use of antihypertensive drugs; (3) triglyceride (TG) $\geqslant$ 150 mg/dL; (4) HDL-C $<$ 40 mg/dL for men and $<$ 50 mg/dL for women; and (5) fasting plasma glucose $\geqslant$ 110 mg/dL or current use of antihyperglycemic drugs. Subjects with a BMI $\geqslant$ 25 kg/m^2 were defined as obese according to the Steering Committee of the World Health Organization Regional Office for the Western Pacific[19]. Subjects with elevated serum alanine aminotransferase (ALT) (ALT $>$ 40 U/L) or aspartate aminotransferase (AST) (AST $>$ 37 U/L) levels were considered to have abnormal liver function. Cardiovascular risk, which is determined by a ratio of total cholesterol (TC)/HDL-C $>$ 5 and correlates significantly with the risk for cardiovascular events[20], was evaluated for its association with erosive esophagitis.

Statistical analysis

Statistical analysis were performed using SPSS (Statistical Package for the Social Sciences) 15 software (SPSS, Chicago, IL, United States). Continuous variables are expressed as the mean ± SD. Student's *t* test was used to compare continuous variables. Univariate analysis was performed using a χ^2 test for categorical variables. For each variable, the OR and 95%CI were calculated. A two-tailed *P* value of $<$ 0.05 was considered statistically significant. Multivariate analysis in the logistic regression model was conducted to examine the associations between erosive esophagitis and different risk factors.

RESULTS

Prevalence of erosive esophagitis

Of the 5031 subjects who underwent upper gastrointestinal endoscopy, 16 were excluded from the analysis because of prior gastric surgery, gastric cancer, or peptic ulcer. Erosive esophagitis was diagnosed in 507 of 5015 subjects. The mean age of subjects with erosive esophagitis was 51.2 ± 11.2 years, and 82.6% were male.

Table 1 Comparison between subjects with and without erosive esophagitis

	With erosive esophagitis (n = 507)	Without erosive esophagitis (n = 507)	P value
Age (yr, mean ± SD)	51.2 ± 11.2	51.2 ± 11.2	-
Sex (M/F)	419/88	419/88	-
BMI (kg/m^2)	25.6 ± 3.6	24.9 ± 3.4	< 0.001
Waist circumference (cm)	87.3 ± 12.9	84.8 ± 11.9	< 0.001
Systolic BP (mmHg)	132.3 ± 18.1	129.2 ± 17.7	0.003
Diastolic BP (mmHg)	87.2 ± 11.7	84.2 ± 10.8	< 0.001
Fasting plasma glucose (mg/dL)	105.9 ± 36.5	101 ± 29	0.009
Triglycerides (mg/dL)	165 ± 107.4	137.1 ± 99.5	< 0.001
HDL-C (mg/dL)	50.4 ± 15	52.2 ± 14.5	0.025
TC/HDL-C	4.27 ± 1.26	4.04 ± 1.13	0.001
LDL-C/HDL-C	2.56 ± 0.96	2.46 ± 0.87	0.049
AST (U/L)	31 ± 28.7	27.3 ± 13.4	0.004
ALT (U/L)	37.1 ± 26.4	31.2 ± 21.6	0.005

The P values are based on Student's t test. M: Male; F: Female; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BP: Blood pressure; BMI: Body mass index; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TC: Total cholesterol.

Characteristics of subjects with and without erosive esophagitis

The subject characteristics are summarized in Table 1. When compared with age- and sex-matched controls, BMI, waist circumference, systolic and diastolic BP, fasting plasma glucose, triglyceride levels, AST/glutamate-oxaloacetate transaminase (GOT) and ALT/glutamate-pyruvate transaminase (GPT) levels, the ratio of TC to HDL-C, and the ratio of low-density-lipoprotein cholesterol to HDL-C (LDL-C to HDL-C) were significantly higher and HDL-C was significantly lower in subjects with erosive esophagitis (all $P < 0.05$).

Univariate and multivariate analyses of the associations between erosive esophagitis and risk factors

The results from the univariate and multivariate logistic regression analyses are shown in Table 2. BMI ⩾ 25 kg/m^2 (OR = 1.72; 95%CI: 1.10-1.80), central obesity (OR = 1.60; 95%CI: 1.20-2.14), hypertension (OR = 1.43; 95%CI: 1.11-1.86), hyperglycemia (OR = 1.39; 95%CI 1.07-1.80), hypertriglyceridemia (OR = 1.50; 95%CI: 1.15-1.95), and cardiovascular disease risk factors of TC/HDL-C > 5 (OR = 1.57; 95%CI: 1.17-2.12), AST > 37 U/L (OR = 1.67; 95%CI: 1.14-2.45), and ALT > 40 U/L (OR = 1.40; 95%CI: 1.04-1.90) were significantly associated with erosive esophagitis, according to the univariate analyses. Furthermore, the multivariate logistic regression analysis confirmed the associations of central obesity, hypertension, hypertriglyceridemia, the ratio of TC/HDL-C > 5, and high AST levels with erosive esophagitis (all $P < 0.05$).

Association of erosive esophagitis with metabolic syndrome

Table 3 shows that the presence of metabolic syndrome (⩾ 3 metabolic criteria) was associated with a higher probability of erosive esophagitis than the presence of < 3 metabolic criteria (OR = 1.475; 95%CI: 1.149-1.895). The prevalence of metabolic syndrome was higher in subjects with erosive esophagitis than in those without (47.1% *vs* 37.7%, respectively; $P < 0.005$).

DISCUSSION

In this study, erosive esophagitis was identified in 10.1% of subjects who underwent routine health examinations. Central obesity, hypertension, hypertriglyceridemia, a high TC/HDL-C ratio (TC/HDL-C > 5), and AST > 37 U/L were significantly associated with erosive esophagitis. Previously, Chua *et al*[13] showed that an increase in BMI was related to an increase in erosive esophagitis, but Chung *et al*[14] did not find a significant association between BMI and erosive esophagitis. Our study showed that central obesity, but not BMI, was an independent risk factor for erosive esophagitis.

A possible reason for this finding is that BMI is not a good indicator of the percentage of body fat among Asian populations[21]. Only the visceral component of abdominal fat increases the risk for erosive esophagitis because visceral adipose tissue is strongly associated with elevated serum levels of proinflammatory adipokines, which may play a role in the development of erosive esophagitis[7,14,22]. In addition, central obesity may increase intra-abdominal pressure and decrease lower esophageal sphincter pressure, resulting in esophageal sphincter relaxation with acid reflux, which may lead to esophagitis[22-24].

Studies have also shown that hypertriglyceridemia is associated with erosive esophagitis[13-15], but contrasting results have also been reported[5]. The present study shows that hypertriglyceridemia is a potential risk factor for erosive esophagitis. Although the underlying mechanisms still need to be fully characterized, high dietary fat intake and delay in gastric emptying may increase the risk of erosive esophagitis[25,26].

The association of hyperglycemia and erosive esophagitis is controversial. Moki *et al*[5] demonstrated a positive relationship between erosive esophagitis and hyperglycemia. However, the majority of studies have found that hyperglycemia is not associated with erosive esophagitis[13-15,27]. Our results also indicate that hyperglycemia is not associated with erosive esophagitis. Gastric emptying can be delayed by diabetic autonomic neuropathy, which may promote erosive esophagitis. However, most individuals in our study population were in generally good health, without diabetic autonomic neuropathy, which may explain our finding that hyperglycemia is not associated with erosive esophagitis. The association of hypertension and erosive esophagitis is also controversial. Gudlaugsdottir *et al*[28] suggested that erosive esophagitis was associated with hypertension, but Wu *et al*[29] failed to establish a significant relationship between hypertension and erosive esophagitis. Our study showed that hyperten-

Table 2 Logistic regression analysis of risk factors for erosive esophagitis

	Univariate analysis OR (95%CI)	*P* value	Multivariate analysis OR (95%CI)	*P* value
Obesity[1]	1.72 (1.10-1.80)	< 0.05		0.606
Central obesity[2]	1.60 (1.20-2.14)	< 0.05	1.38 (1.00-1.86)	< 0.05
Hypertension	1.43 (1.11-1.86)	< 0.05	1.35 (1.04-1.76)	< 0.05
Hyperglycemia	1.39 (1.07-1.80)	< 0.05		0.143
Hypertriglyceridemia	1.50 (1.15-1.95)	< 0.05	1.34 (1.02-1.76)	< 0.05
Low HDL-C	1.24 (0.91-1.67)	0.192	-	-
TC/HDL-C > 5	1.57 (1.17-2.12)	< 0.05	1.45 (1.06-1.97)	< 0.05
AST > 37 (U/L)	1.67 (1.14-2.45)	< 0.05	1.59 (1.08-2.34)	< 0.05
ALT > 40 (U/L)	1.40 (1.04-1.90)	< 0.05		0.986

[1]Defined as body mass index ⩾ 25 kg/m^2; [2]Defined as waist circumference ⩾ 90 cm for men and ⩾ 80 cm for women. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HDL-C: High-density lipoprotein cholesterol; TC: Total cholesterol.

Table 3 Components of metabolic syndrome associated with erosive esophagitis *n* (%)

Metabolic factors	Erosive esophagitis (*n* = 507)	Matched normal control (*n* = 507)	OR (95%CI)	*P* value
Number of criteria				
⩾ 1 criterion	473 (93.3)	447 (88.2)	1.867 (1.203-2.899)	0.007
⩾ 2 criteria	391 (77.1)	335 (66.1)	1.731 (1.312-2.283)	< 0.001
⩾ 3 criteria	239 (47.1)	191 (37.7)	1.475 (1.149-1.895)	0.003
⩾ 4 criteria	112 (22.1)	64 (12.6)	1.963 (1.403-2.746)	< 0.001
5 criteria	28 (5.5)	20 (3.9)	1.423 (0.791-2.561)	0.301

sion is an independent risk factor for erosive esophagitis.

Several studies have reported that abnormal liver function is related to metabolic syndrome[10-12]. In addition to metabolic syndrome, high BMI, central obesity, and hyperlipidemia are associated with hepatic steatosis[30,31]. The severity of hepatic steatosis is significantly correlated with the results of hepatic enzyme tests[32]. Several studies have reported an association between gastroesophageal reflux disease (GERD) and chronic liver disease[33-36]. Suzuki *et al*[36] showed that more than 30% of patients with chronic liver disease had GERD. Ueda *et al*[34] showed a relatively higher incidence of GERD in patients with alcoholic liver disease. We found that abnormal liver function (higher AST level) was a risk factor for erosive esophagitis. Although the underlying mechanisms are not clear, hepatic steatosis related to metabolic syndrome, a risk factor of erosive esophagitis, may provide a potential explanation. The TC/HDL-C ratio has been used to predict the risk of future coronary heart diseases. This practice is supported by several studies that have demonstrated that the TC/HDL-C ratio is the most significant predictor of future coronary heart disease, along with smoking and diabetes mellitus[37-39]. Our study showed that subjects with erosive esophagitis might have a higher cardiovascular risk than those without erosive esophagitis. The present study has several limitations. First, only the association between abnormal liver function and erosive esophagitis was analyzed, and no further evaluation was conducted to determine the relationship between erosive esophagitis and hepatic steatosis or chronic hepatitis B or C. Second, this study had a cross-sectional design, and therefore, only the associations between erosive esophagitis and metabolic syndrome, abnormal liver function, and dyslipidemia could be determined. Further studies with a longitudinal design are required to evaluate their possible causal relationships.

In conclusion, metabolic syndrome and its components, such as central obesity, BP, and hypertriglyceridemia, are independent risk factors for erosive esophagitis. Abnormal liver function, including elevated AST, and cardiovascular risk factors were also found to be associated with erosive esophagitis. Further studies are needed to investigate the underlying mechanisms responsible for the relationship between abnormal liver function, cardiovascular risk, and erosive esophagitis.

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COMMENTS

Background

Erosive esophagitis is becoming more prevalent in Asia, but the underlying mechanism remains unknown. Obesity has been associated with erosive esophagitis. However, the associations of metabolic syndrome, its components, and liver function with erosive esophagitis are controversial.

Research frontiers

Several previous studies have identified the risk factors of erosive esophagitis, including male sex, hiatal hernia, smoking, alcohol consumption, and obesity. This study further determined the associations between erosive esophagitis and metabolic syndrome, its components, and liver function.

Innovations and breakthroughs

The present study demonstrated that metabolic syndrome, impaired liver function, and dyslipidemia were associated with erosive esophagitis.

Applications

Individuals with metabolic syndrome and high cardiovascular risk, as defined by

a higher ratio of total cholesterol to high-density lipoprotein cholesterol (HDL-C), are at higher risk for erosive esophagitis. This finding may suggest that treating metabolic disorders can prevent or reduce the risk of erosive esophagitis. However, further studies are needed to confirm this finding.

Terminology

Metabolic syndrome is a complex disorder comprising central obesity, high blood pressure, hyperglycemia, hypertriglyceridemia, and a low concentration of HDL-C. Cardiovascular risk, which is determined by a total cholesterol/HDL-C ratio > 5, correlates with the risk of cardiovascular events.

Peer review

Authors undertook to characterize the correlation between erosive esophagitis and metabolic syndrome and its components, abnormal liver function, and abnormal lipoprotein profiles. This case-controlled study is well organized and has enough potential for publication.

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BRIEF ARTICLE

Effect of early enteral combined with parenteral nutrition in patients undergoing pancreaticoduodenectomy

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Abstract

AIM: To investigate the effect of early enteral nutrition (EEN) combined with parenteral nutritional support in patients undergoing pancreaticoduodenectomy (PD).

METHODS: From January 2006, all patients were given EEN combined with parenteral nutrition (PN) (EEN/PN group, n = 107), while patients prior to this date were given total parenteral nutrition (TPN) (TPN group, n = 67). Venous blood samples were obtained for a nutrition-associated assessment and liver function tests on the day before surgery and 6 d after surgery. The assessment of clinical outcome was based on postoperative complications. Follow-up for infectious and noninfectious complications was carried out for 30 d after hospital discharge. Readmission within 30 d after discharge was also recorded.

RESULTS: Compared with the TPN group, a significant decrease in prealbumin (PAB) (P = 0.023) was seen in the EEN/PN group. Total bilirubin (TB), direct bilirubin (DB) and lactate dehydrogenase (LDH) were significantly decreased on day 6 in the EEN/PN group (P = 0.006, 0.004 and 0.032, respectively). The rate of grade Ⅰ complications, grade Ⅱ complications and the length of postoperative hospital stay in the EEN/PN group were significantly decreased (P = 0.036, 0.028 and 0.021, respectively), and no hospital mortality was observed in our study. Compared with the TPN group (58.2%), the rate of infectious complications in the EEN/PN group (39.3%) was significantly decreased (P = 0.042). Eleven cases of delayed gastric emptying were noted in the TPN group, and 6 cases in the EEN/PN group. The rate of delayed gastric emptying and hyperglycemia was significantly reduced in the EEN/PN group (P = 0.031 and P = 0.040, respectively).

CONCLUSION: Early enteral combined with PN can greatly improve liver function, reduce infectious complications and delayed gastric emptying, and shorten postoperative hospital stay in patients undergoing PD.

Key words: Enteral nutrition; Parenteral nutrition; Pancreaticoduodenectomy; Complications; Metabolism

Core tip: On the basis of our experience and the findings of previous studies, we investigated the effect of early enteral nutrition combined with parenteral nutritional support in patients undergoing pancreaticoduodenectomy enrolled in a retrospective controlled clinical trial. The results of this study showed that early enteral nutritional support combined with parenteral nutrition can greatly improve nutritional status and liver function, decrease the incidence of infectious complications and delayed gastric emptying, and shorten the length

of postoperative hospital stay.

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INTRODUCTION

Pancreaticoduodenectomy (PD) is currently considered the treatment of choice for carcinoma of the periampullary region. Patients who are candidates for PD often have associated comorbidities such as diabetes, jaundice, and protein-energy malnutrition. PD results in loss of the gastric pacemaker and a partial pancreatic resection, and the physiologic consequence of this is a high incidence of postoperative malnutrition. PD is associated with a high incidence of postoperative complications, and this high rate of complications is likely to be multifactorial and may include overall nutritional debilitation[1]. Postoperative nutritional support therapy could ameliorate the clinical outcome in many types of surgical treatment, diminish the incidence of postoperative complications, and may be important in patients undergoing PD.

Recent research has shown that early postoperative enteral nutrition (EN) enhanced immunocompetence, decreased clinical infection rates, maintained gut structure and function, and can potentially attenuate catabolic stress responses in patients after surgery[2,3]. In addition, EN is believed to be safer and less expensive than parenteral nutrition (PN). However, postoperative total enteral feeding is associated with complications such as diarrhea, abdominal distention, and abdominal cramps. These symptoms worsen with increasing caloric intake and can lead to discontinuance of enteral feeding[2,4]. Gastroparesis is a frequent postoperative event following PD resection, and this often necessitates prolonged gastric decompression and enteral nutritional support[5]. Clinical data on postoperative early enteral nutrition (EEN) after PD are very limited. Therefore, on the basis of our experience and the findings of previous studies, we investigated the effect of EEN combined with parenteral nutritional support in patients undergoing PD enrolled in a retrospective controlled clinical trial.

MATERIALS AND METHODS

Patient selection

From January 2004 to June 2011, 196 patients underwent PD due to peri-ampullary tumors in the Department of Hepatobiliary Surgery at the Affiliated Drum Tower Hospital of Medical School of Nanjing University, China, where the authors work. Nineteen patients with manifest metabolic diseases (*e.g.*, diabetes mellitus and hyperthyroidism), severe hemorrhagic disease, ongoing infection, inflammatory bowel diseases or severe renal abnormality were excluded. Three patients with a history of gastric or pancreatic resection were also excluded, given the possible influence this procedure may have on the incidence of delayed gastric emptying. From January 2006, all patients were given EEN combined with PN (EEN/PN group, n = 107), while patients prior to this date were given TPN (TPN group, n = 67).

The primary endpoint of this study was the occurrence of major complications, and the secondary endpoint was 30 d after hospital discharge. The Nutrition Risk Screening 2002 (NRS 2002) scoring system[6] was used in this study, and the post-operative NRS 2002 score in all patients was $\geqslant$ 3, indicating that all patients required nutritional support.

Treatment

TPN was given 24 h/d for 5 d from the first day after PD. The nitrogen intake was 0.25 g/kg body weight per day, caloric intake was 125.4 kJ/kg per day and lipid intake was 1.1 g/kg per day. The nonprotein calories were given as dextrose (5.0 g/kg per day) and fat emulsion in a ratio of 2:1. The source of lipids was the standard lipid emulsion (20% emulsion, 5.5 mL/kg per day, long chain triglycerides: medium chain triglycerides 1:1, Huarui Pharmaceuticals, Jiangsu Province, China). Patients received 1.5 g amino acids/kg per day, administered as a commercially available compound amino acid solution (20% solution, Huarui Pharmaceuticals, Jiangsu Province, China). The proportion of nonprotein calories with nitrogen in both groups was 501.6 kJ/g. The PN solutions were prepared by a clinical pharmacist under aseptic conditions and adjusted to the weight of each patient. The amino acids, fat emulsion and dextrose mixture with electrolytes, vitamins and trace elements were administered *via* a central venous catheter. As soon as bowel function returned on 3-4 d after surgery, all patients were given liquid carbohydrate and cow's milk protein in equal amounts orally.

The surgical treatment was standardized, and lymph-node dissection was performed according to the definition provided by Pedrazzoli *et al*[7]. PD was performed by three groups of surgeons using the same technique. All patients received the same antibiotics postoperatively.

Patients in the EEN/PN group underwent preoperative placement of a conventional gastric tube. When gastrojejunostomy was complete, nasojejunal nutrition tubes were positioned (10 F, NUTRICIA Pharmaceutical Co., The Netherlands) from the nasal cavity to the output loops of the jejunum (approximately 20-25 cm with the help of a surgeon). The jejunum nutrition tube filar guide was then removed when the tube was in the correct position.

EN was given to patients in the EEN/PN group 24 h/d. An infusion of 100 mL of 5% glucose and sodium chloride injection (GNS) *via* a nasojejunal feeding tube was commenced within 24 h of surgery and 500 mL of 5% GNS was given on post-operative day 2 (POD2). On

Table 1 Ranges and averages of calories, protein, fat and carbohydrates in the enteral and parenteral regimens in the early enteral nutrition/parenteral nutrition group

POD	Nutritional support	Calories (kJ)	Protein (g)	Fat (g)	Carbohydrates (g)
1	PN	7649.4 (6081.9-10282.8)	91.5 (72.8-123.0)	67.1 (53.3-90.2)	305.0 (242.5-410.0)
	EN	83.6	0	0	5
2	PN	7649.4 (6081.9-10282.8)	91.5 (72.8-123.0)	67.1 (53.3-90.2)	305.0 (242.5-410.0)
	EN	418	0	0	25
3	PN	6447.6 (4880.1-9081.0)	84.0 (65.3-115.5)	59.6 (45.8-82.7)	257.5 (195.0-362.5)
	EN	1201.8	7.5	7.5	47.5
4	PN	5559.4 (3991.9-8192.8)	76.5 (57.8-108.0)	52.1 (38.3-75.2)	235.0 (172.5-340.0)
	EN	2090	15	15	70
5	PN	3469.0 (1901.9-6102.8)	61.5 (42.8-93.0)	37.1 (23.3-60.2)	165.0 (102.5-270.0)
	EN	4180	30	30	140
6	PN	0	0	0	0
	EN	7649.4 (6081.9-10282.8)	54.9 (43.65-73.8)	54.9 (43.65-73.8)	256.2 (203.7-344.4)

Data are expressed as absolute average or average (range). POD: Post-operation day; PN: Parenteral nutrition; EN: Enteral nutrition.

POD3, 250 mL of Peptisorb liquid (2092 kJ/500 mL, NUTRICIA Pharmaceutical Co., the Netherlands) and 250 mL of 5% GNS were administered. Patients received 500 mL of Peptisorb liquid on POD4, and 1000 mL on POD5. From POD3, the PN recipe was adjusted according to the amount of EN, and the total caloric intake of PN and EN was 125.4 kJ/kg per day. PN was stopped on POD6, and patients in the EEN/PN group reached a maximum volume of total caloric intake following Peptisorb liquid (30 mL/kg per day). Oral intake started on POD7 and EN was stopped when the patients tolerated an intake of over 1000 kcal/d.

The body weight of the patients in this study varied from 48.1-84.6 kg, with an average body weight of 60.3 kg. Based on the range and average body weight, the ranges and averages of the calories, protein, fat and carbohydrates in the enteral and parenteral regimens in the EEN/PN group are listed in Table 1.

Assessment

Venous heparin blood samples were obtained on 1 d (the day before surgery), and 6 d after surgery. Three types of measurement were carried out. First, a nutrition-associated assessment was carried out, which included serum albumin, prealbumin (PAB), total protein (TP), transferrin (TF) and total lymphocyte counts (TLCs). Serum albumin, PAB, total protein and TF were determined by an automatic biochemistry analyzer (HITACHI 7600, Hitachi Co., Tokyo, Japan). TLCs were determined using an automatic blood cell analyzer (COULTER STKS). The prognostic nutritional index (PNI) was calculated as follows: PNI = 0.005 × TLC (10^6/L) + albumin (g/L). The normal value of PNI is more than 50, and PNI values < 40 indicated malnutrition. Nitrogen balance was calculated as follows: N balance (g N/d) = [protein intake (g/d)/6.25] - [urinary urea (g/24 h)/2.14 + 3 g (nitrogen lost in skin and stool per day)]. Second, a liver function assessment was carried out, which included serum total bilirubin (TB), direct bilirubin (DB), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) measurements. Liver function was determined by an automatic biochemistry analyzer (HITACHI 7600). Finally, clinical outcome was assessed based on postoperative complications. These complications were graded according to the Clavien-Dindo classification[8], which was validated in pancreatic surgery[9]. Complications graded as Ⅲ to Ⅴ were considered as major. Pancreatic fistula and delayed gastric emptying were defined according to the International Study Group of Pancreatic Surgery (ISGPS)[10,11]. Operative mortality was defined as in-hospital death or death occurring within 30 d of discharge. Follow-up for infectious and noninfectious complications was carried out for 30 d after hospital discharge. Readmission within 30 d after discharge was also recorded.

Statistical analysis

The results are expressed as mean ± SD. Data were analyzed using the Statistical Analysis System (SAS). Differences between means were evaluated using the Student *t* test when normal distribution was confirmed by the Shapiro-Wilks test. When the hypothesis of normal distribution was rejected, differences between groups were tested by nonparametric statistics using the Mann-Whitney test for unpaired samples and Wilcoxon criteria for paired samples. Fisher's exact test was used for analysis of categorical values when appropriate. A P value of < 0.05 was considered significant.

RESULTS

A total of 174 patients were enrolled in the study, 67 patients in the TPN group and 107 patients in the EEN/PN group. The mean age of the subjects was 53.2 years (range, 37-68 years). Demographic and preoperative clinical data, including age, sex, preoperative hemoglobin, preoperative albumin and the number of patients with jaundice or preoperative endoscopic nasal biliary drainage, are summarized in Table 2. No significant differences with respect to intraoperative factors, including opera-

Table 2 Preoperative clinical data, intraoperative factors and histopathology of the patients enrolled in the study

	TPN group	EEN/PN group
Sex (male/female)	44/23	70/37
Age (yr)	52. 8 ± 11.2	53.9 ± 10.6
Intraoperative factors		
Patients with jaundice (%)	79.1	83.2
Patients with preoperative ENBD (%)	50.7	48.6
Preoperative hemoglobin (g/L)	11.8 ± 1.0	12.4 ± 0.8
Preoperative albumin (g/L)	37.9 ± 3.1	36.8 ± 3.6
Duration of surgery (min)	345.1 ± 64.8	332.7 ± 56.6
Operative blood loss (mL)	648.4 ± 262.6	680.2 ± 193.7
Blood transfusion (%)	26.9	31.7
Histopathologic finding (*n*)		
Pancreatic head carcinoma	24	37
Distal cholangiocarcinoma	19	31
Periampullary adenocarcinoma	21	34
Duodenal adenocarcinoma	3	5

EEN: Early enteral nutrition; ENBD: Endoscopic nasal biliary drainage; PN: Prognostic nutritional; TPN: Total parenteral nutrition.

tion time, blood loss, number of patients who received blood transfusion and histopathological diagnosis, were observed between the two groups ($P > 0.05$).

Nutrition-associated assessment

No significant difference in the pre-operative nutrition-associated assessment was seen between the two groups. Compared with the results on the day before PD, a decrease in TP, PAB, TF and PNI was observed on day 6 after PD in all patients in this study, and a significant decrease in PAB in the TPN group ($P < 0.05$) with no significant difference ($P > 0.05$) in the EEN/PN group (Table 3).

Compared with the TPN group, a significant decrease in PAB ($P = 0.02$) was seen in the EEN/PN group. However, no significant differences in TF, TP and PNI were noted between the two groups ($P > 0.05$). Nitrogen balance was negative in both groups on day 6, with no significant difference between the two groups (Table 3).

Liver function assessment

No significant differences in pre-operative liver function assessment were seen between the two groups. Compared with the results on the day before surgery, a significant decrease in ALT, AST, TB, DB and LDH was observed on 6 d in both groups ($P < 0.05$), and a very significant decrease in TB and DB in the EEN/PN group ($P < 0.01$).

Compared with the TPN group, a significant decrease in TB, DB and LDH was seen in the EEN/PN group ($P < 0.05$). No significant differences in ALT and AST were observed between the two groups ($P > 0.05$; Table 4).

Clinical outcome

A prognostic score for major morbidity after PD has recently been proposed by Braga *et al*[12]. The predictive risk score of major complications after PD in the two groups are listed in Table 5. There were no significant differences between the two groups in the score categorized in 4 risk classes ($P > 0.05$).

Table 6 shows the postoperative outcome in the two groups. Reoperation was necessary in 9 patients, and the causes of reoperation were early bleeding (1 case in the TPN group and 2 cases in the EEN/PN group), late bleeding (1 case in the EEN/PN group), abdominal abscess (2 cases in the TPN group and 2 cases in the EEN/PN group) and intestinal obstruction (1 case in the EEN/PN group). The causes of readmission in this study were intestinal obstruction (1 case in the TPN group and 2 cases in the EEN/PN group) and cholangitis (1 case in the TPN group and 1 case in the EEN/PN group). The rate of grade I complications, grade Ⅱ complications and the length of postoperative hospital stay in the EEN/PN group were significantly reduced ($P < 0.05$), and no hospital mortality was observed in this study (Table 6).

Postoperative complications are shown in detail in Table 7. There were 39 cases of infectious complications in the TPN group (8 cases of pneumonia, 7 cases of abdominal abscess, 5 cases of bile leak, 2 cases of pancreatic fistula, 4 cases of cholangitis, 8 cases of wound infection and 5 cases of urinary tract infection) and 42 cases in the EEN/PN group (6 cases of pneumonia, 6 cases of abdominal abscess, 7 cases of bile leak, 4 cases of pancreatic fistula, 3 cases of cholangitis, 10 cases of wound infection and 6 cases of urinary tract infection). Compared with the TPN group (58.2%), the rate of infectious complications in the EEN/PN group (39.3%) was significantly decreased ($P < 0.05$). Eleven cases of delayed gastric emptying were observed in the TPN group, and 6 cases in the EEN/PN group. The rate of delayed gastric emptying and hyperglycemia was significantly decreased in the EEN/PN group ($P < 0.05$). There were 29 cases of enteral-feeding-related complications in the EEN/PN group, including diarrhea, abdominal distention, and abdominal cramps. These symptoms were alleviated by slowing down the speed of enteral transfusion or by the administration of medications. None of the patients discontinued enteral feeding, and no enteral-feeding-related complications were noted in the TPN group.

DISCUSSION

PD is associated with a high incidence of postoperative complications, and an overall morbidity rate of 48% can be anticipated at major centers[13]. The high rate of complications is likely to be multifactorial and may include overall nutritional debilitation, as most patients with periampullary tumors present with significant weight loss due to anorexia and malabsorption, and are expected to have a period of inadequate oral intake up to 10 d after surgery[14]. Compared with the results on the day before PD, a decrease in TP, PAB, TF, PNI and negative nitrogen balance were observed on day 6 in all patients in this study. Perioperative nutritional support can be beneficial in these patients in that it may reduce mortality and morbidity, and the length of hospital stay[15].

Numerous studies have suggested that EN has sev-

Table 3 Comparison of nutrition-associated assessment in the two groups (mean ± SD)

	Normal value	Group	Day 1	Day 6	Decrease (days 1-6)
TP (g/L)	62-85	TPN	63.46 ± 7.24	59.92 ± 7.65	3.54 ± 1.72
		EEN/PN	64.11 ± 6.84	61.12 ± 6.83	2.99 ± 1.07
PAB (mg/L)	0-800	TPN	196.25 ± 64.32	116.52 ± 72.16[a]	79.73 ± 35.32
		EEN/PN	190.15 ± 62.18	158.32 ± 62.46	31.83 ± 13.15[c]
TF (g/L)	2.2-12	TPN	2.53 ± 0.76	2.20 ± 0.72	0.33 ± 0.61
		EEN/PN	2.46 ± 0.68	2.08 ± 0.81	0.38 ± 0.72
PNI	> 50	TPN	50.36 ± 9.14	43.12 ± 8.13	7.24 ± 7.40
		EEN/PN	51.62 ± 8.16	45.15 ± 9.52	6.47 ± 5.93
N-balance (g/d)		TPN	/	-(14.76 ± 6.03)	/
		EEN/PN	/	-(15.91 ± 7.85)	/

[a]$P < 0.05$ *vs* day 1; [c]$P < 0.05$ *vs* total parenteral nutrition (TPN) group. PAB: Prealbumin; PNI: Prognostic nutritional index; TP: Total protein; TF: Transferrin; EEN: Early enteral nutrition; PN: Parenteral nutrition.

Table 4 Comparison of liver function in the two groups (mean ± SD)

	Normal value	Group	Day 1	Day 6	Decrease (days 6-1)
ALT (μ/L)	5-40	TPN	138.2 ± 48.4	82.5 ± 42.3[a]	55.7 ± 31.5
		EEN/PN	145.1 ± 39.2	77.4 ± 37.6[a]	67.7 ± 36.2
AST (μ/L)	8-40	TPN	97.6 ± 36.2	55.1 ± 31.5[a]	42.5 ± 26.2
		EEN/PN	102.3 ± 41.3	63.2 ± 36.3[a]	39.1 ± 22.0
TB (μmol/L)	5-20.5	TPN	112.5 ± 37.5	66.2 ± 29.4[a]	46.3 ± 34.3
		EEN/PN	106.8 ± 36.2	41.5 ± 34.1[b]	65.3 ± 36.2[c]
DB (μmol/L)	1.7-6.8	TPN	78.6 ± 30.2	38.1 ± 26.2[a]	40.5 ± 21.3
		EEN/PN	81.7 ± 35.6	22.4 ± 16.2[b]	59.3 ± 28.1[c]
LDH (μ/L)	109-245	TPN	332.6 ± 89.4	264.3 ± 101.3[a]	68.3 ± 51.2
		EEN/PN	316.2 ± 98.1	211.5 ± 86.2[a]	104.7 ± 76.8[c]

[a]$P < 0.05$, [b]$P < 0.01$ *vs* day 1; [c]$P < 0.05$ *vs* total parenteral nutrition (TPN) group. PN: Prognostic nutritional; EEN: Early enteral nutrition; TB: Total bilirubin.

Table 5 Predictive risk score of major complications after pancreaticoduodenectomy in the two groups

Predictor	Categories	Risk Score	TPN group	EEN/PN group
Pancreatic texture (%)	Hard	0	43	73
	Soft	4	24	34
Pancreatic duct diameter (%)	> 3 mm	0	48	81
	≤ 3 mm	1	19	26
Operative blood loss (%)	< 700 mL	0	55	81
	≥ 700 mL	4	12	26
ASA score (%)	I	0	31	55
	II	2	33	47
	III	6	3	5
Score categorized in 4 risk classes *n* (%)	0-3		23 (34.3)	38 (35.5)
	4-7		22 (32.8)	33 (30.8)
	8-11		19 (28.4)	31 (29.0)
	12-15		3 (4.5)	5 (4.7)

ASA: American Society of Anesthesiologist; EEN: Early enteral nutrition; TPN: Total parenteral nutrition; PN: Prognostic nutritional.

Table 6 Postoperative outcome in the two groups *n* (%)

Group	TPN group	EEN/PN group
Complication Grade		
No complications	24 (35.8)	42 (39.2)
I	33 (49.3)	38 (35.5)[a]
II	38 (56.7)	42 (39.3)[a]
IIIa	7 (10.4)	10 (9.3)
IIIb	4 (6.0)	5 (4.6)
IVa	0 (0.0)	0 (0.0)
IVb	0 (0.0)	0 (0.0)
V (mortality)	0 (0.0)	0 (0.0)
Reoperation	3 (4.5)	6 (5.6)
Readmission	2 (3.0)	3 (2.8)
Postoperative hospital stay (d)	16.8 ± 6.2	13.2 ± 4.7[a]

Numbers of single types of complications do not add up to the number of patients within the 2 groups, due to the possible occurrence of more types of complications in some patients. [a]$P < 0.05$ *vs* total parenteral nutrition (TPN) group. EEN: Early enteral nutrition; PN: Prognostic nutritional.

eral advantages over TPN. Early enteral feeding was shown to reduce postoperative septic complications in a meta-analysis of 8 prospective randomized trials, and improve glucose tolerance, protein kinetics and wound healing. Furthermore, EN is safer and less expensive than PN[16,17]. However, postoperative total enteral feeding is associated with complications such as diarrhea, abdominal distention, and abdominal cramps. These symptoms worsen with increasing caloric intake and can lead to discontinuance of enteral feeding[2,3]. On the basis of these findings, we considered EEN combined with PN to be a better mode of postoperative nutritional support than total enteral feeding. On the first three days after surgery in this study, the amount of EN increased slowly to avoid severe gastrointestinal complications. Twenty-

Table 7 Postoperative complications in the two groups *n* (%)

Complications	TPN group	EEN/PN group
Pancreatic fistula	2 (3.0)	4 (3.7)
Grade A	0 (0.0)	0 (0.0)
Grade B	2 (3.0)	4 (3.7)
Grade C	0 (0.0)	0 (0.0)
Wound infection	8 (11.9)	10 (9.3)
Abdominal abscess	7 (10.4)	6 (5.6)
Bile leak	5 (7.5)	7 (6.5)
Cholangitis	4 (6.0)	3 (2.8)
Urinary tract infection	5 (7.5)	6 (5.6)
Pneumonia	8 (11.9)	6 (5.6)
Catheter-related sepsis	0 (0.0)	0 (0.0)
Gastrointestinal bleeding	4 (6.0)	5 (4.7)
Intraperitoneal bleeding	3 (4.5)	6 (5.6)
Delayed gastric emptying	11 (16.4)	6 (5.6)
Enteral-feeding-related complications	0 (0.0)	29 (27.1)
Abdominal cramps	0 (0.0)	6 (5.6)
Abdominal distention	0 (0.0)	11 (10.3)
Diarrhea	0 (0.0)	9 (8.4)
Vomiting	0 (0.0)	3 (2.8)
Hyperglycemia	12 (17.9)	6 (5.6)

Numbers of single types of complications do not add up to the number of patients within the 2 groups, due to the possible occurrence of more types of complications in some patients. EEN: Early enteral nutrition; PN: prognostic nutritional; TPN: Total parenteral nutrition.

nine cases in the EEN/PN group had enteral-feeding-related complications, these symptoms were alleviated by slowing down the speed of enteral transfusion or by the administration of medications, and none of the patients discontinued enteral feeding or dropped out of the study. PAB, which is more sensitive than albumin for evaluating protein synthesis in the liver due to its shorter half-life, was decreased on day 6 in all patients in this study. Compared with the TPN group, a significant decrease in PAB ($P < 0.05$) was observed in the EEN/PN group.

Changes in transaminase and bilirubin are the most important indices for evaluating liver function in patients after PD. All patients in this study underwent PD to remove biliary obstruction, therefore, ALT, AST, TB, DB and LDH were significantly reduced. Lack of enteral feeding has several metabolic and endocrine consequences on intestinal and liver function. Experimental studies have shown that the fasted state reduces the secretion of several gastrointestinal hormones, such as cholecystokinin, gastrin and peptide YY. These hormones are instrumental in stimulating bile flow and gallbladder contraction, and for maintaining intestinal motility[18-20]. EN can also stimulate hepatic circulation and ameliorate liver function[21]. In the present study, a significant decrease in TB and DB in the EEN/PN group was observed compared with that in the TPN group.

EN preserved the gut flora architecture, prevented gastrointestinal mucosa atrophy, and inhibited microbial translocation from the gut to the blood stream[22,23]. Compared with the TPN group, the rate of infectious complication in the EEN/PN group was significantly decreased. The reduced length of postoperative hospital stay in the EEN/PN group indicated that the time to complete recovery could be shortened by EEN support combined with PN. This may be explained by the lower number of complications.

Delayed gastric emptying (DGE) is also known as "gastroparesis". DGE is not a fatal complication, but sometimes results in a significant prolongation of hospital stay and increased hospital costs. DGE has been reported to be affected by several factors including gastric dysrhythmias due to intra-abdominal complications, gastric atony after duodenal resection in response to a reduction in motilin levels, pylorospasm secondary to vagotomy, and angulation of the reconstructed alimentary tract[24-26]. Eleven cases of DGE were observed in the TPN group, and 6 cases in the EEN/PN group. EEN-support therapy significantly decreased the rate of DGE. One potential mechanism for the decreased rate of DGE due to EN may be the mechanical effects caused by the nasojejunal tube or simply its presence across the anastomosis, which stimulates the motility of the stomach and jejunum, while another mechanism may be the stimulation of bowel movements by the input of nutritional liquids[27,28].

In conclusion, we have shown that early enteral nutritional support combined with PN can greatly improve nutritional status and liver function, decrease the incidence of infectious complications and delayed gastric emptying, and shorten postoperative hospital stay in patients undergoing PD. Future randomized controlled trials are necessary to identify the correct application of PN and EN in patients receiving PD.

COMMENTS

Background

Pancreaticoduodenectomy (PD) is associated with a high incidence of postoperative complications. This high rate of complications is likely to be multifactorial and may include overall nutritional debilitation. Postoperative nutritional support therapy could ameliorate the clinical outcome in many types of surgical treatment and diminish the incidence of postoperative complications. The clinical data on postoperative early enteral nutrition (EEN) combined with parenteral nutrition (PN) after PD is very limited.

Research frontiers

Recent research has shown that early postoperative enteral nutrition enhanced immunocompetence, lowered clinical infection rates, and maintained gut structure and function, and can potentially attenuate catabolic stress responses in patients after surgery. However, postoperative total enteral feeding is associated with complications such as diarrhea, abdominal distention, and abdominal cramps. These symptoms worsened with increasing caloric intake and can lead to discontinuance of enteral feeding.

Innovations and breakthroughs

The authors investigated the effect of EEN combined with parenteral nutritional support in patients undergoing PD enrolled in a retrospective controlled clinical trial on the basis of their experience and the findings of previous studies. The results of this study show that early enteral nutritional support combined with PN can greatly improve nutritional status and liver function, decrease the incidence of infectious complications and delayed gastric emptying, and shorten postoperative hospital stay.

Applications

The results of this study show that early enteral nutritional support combined with PN can greatly improve nutritional status and liver function, decrease

the incidence of infectious complications and delayed gastric emptying, and shorten postoperative hospital stay in patients undergoing PD. These findings are clinically relevant for guiding surgeons in the perioperative administration of medications during PD.

Peer review

This is an interesting study which is well written and referenced. It is a non-randomized retrospective study of the effect of EEN combined with parenteral nutritional support for patients receiving PD. PD is a major surgical procedure for the treatment of periampullary tumors which will result in a high incidence of complications and postoperative malnutrition, but nutritional support can improve patient's malnutrition and diminish the incidence of postoperative complications.

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BRIEF ARTICLE

Association between *UCP3* gene polymorphisms and nonalcoholic fatty liver disease in Chinese children

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Abstract

AIM: To confirm the hypothesis that polymorphisms of the uncoupling protein 3 (*UCP3*) gene are associated with the occurrence of nonalcoholic fatty liver disease (NAFLD).

METHODS: A total of 250 NAFLD patients (147 males and 103 females) and 200 healthy individuals who served as controls (control, 109 males and 91 females), aged between 6 and 16 years were enrolled in this study. The four non-synonymous single nucleotide polymorphisms (SNPs) in the *UCP3* gene polymorphisms of rs1726745, rs3781907, rs11235972 and rs1800849, were genotyped using MassArray. Body mass index (BMI), waist and hip circumference, blood pressure (BP), fasting blood glucose (FBG), insulin and lipid profiles were measured and B-ultrasound examination was performed in all subjects.

RESULTS: NAFLD patients showed risk factors for metabolic syndrome: elevated BMI, waist-to-hip ratio, BP, FBG, homeostasis model assessment-estimated insulin resistance, total triglyceride, total cholesterol and low-density lipoprotein-cholesterol, while decreased high-density lipoprotein-cholesterol level compared with the control group. The GG genotype distributions of rs11235972 in the NAFLD group differed significantly from that in the control group. We found that waist circumference between CC (58.76 ± 6.45 cm) and CT+TT (57.00 ± 5.59 cm), and hip circumference between CC (71.28 ± 7.84 cm) and CT+TT genotypes (69.06 ± 7.75 cm) were significantly different with and without rs1800849 variation ($P < 0.05$).

CONCLUSION: A higher prevalence of rs11235972 GG genotype was observed in the NAFLD group compared with the control group. No differences were observed for the other SNPs. However, there was a significant difference in body height in addition to waist and hip circumference between the CC (mutant type group) and CT+TT group with and without rs1800849 variation.

Key words: Nonalcoholic fatty liver disease; Uncoupling protein 3; Single nucleotide polymorphisms

Core tip: There are few population-based prevalence

studies of pediatric nonalcoholic fatty liver disease (NAFLD). Uncoupling protein 3 (*UCP3*) is considered to be associated with obesity, given the role for *UCP3* in the regulation of energy and lipid metabolism. This is the first study to report that there significant difference of body height, waist and hip circumference between CC (mutant type group) and CT+TT group with and without rs1800849 variation were found. This study confirmed the hypothesis that polymorphisms of the *UCP3* are associated with the occurrence of NAFLD. These variations could be useful for the diagnosis and/or prognosis of NAFLD.

Xu YP, Liang L, Wang CL, Fu JF, Liu PN, Lv LQ, Zhu YM. Association between *UCP3* gene polymorphisms and nonalcoholic fatty liver disease in Chinese children. *World J Gastroenterol* 2013; 19(35): 5897-5903 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5897.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5897

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathologic condition characterized by abnormal lipid deposition in hepatocytes in the absence of excess alcohol intake. NAFLD comprises a wide spectrum of liver damage, including simple steatosis, steatohepatitis, fibrosis or even cirrhosis of the liver[1]. NAFLD does not only impact adults, but is also one of the major causes of liver diseases in children[2]. There are few population-based prevalence studies of pediatric NAFLD. Some studies have suggested a prevalence of 2.6%-9.6% for suspected NAFLD among children and adolescents in the United States[3,4] and Asia[5,6]. NAFLD has been shown to be associated with metabolic syndrome (MetS), which comprises obesity, type 2 diabetes, dyslipidemia and high blood pressure (BP) with insulin resistance being the central mechanism[7,8]. Theoretically, many variations in candidate genes related to MetS may contribute to the pathogenesis of NAFLD, such as genes related to insulin resistance and genes influencing hepatic free fatty acid metabolism. Elucidation of genetic factors that predispose an individual to NAFLD may lead to the development of non-invasive biomarkers for the early diagnosis of NAFLD and may allow early preventive and therapeutic strategies for those at the high risk.

Uncoupling protein 3 (*UCP3*) gene is located on chromosome 11q13. *UCP3* is a mitochondrial anion carrier protein with a highly selective expression in skeletal muscle, a major site of thermogenesis in humans, which makes an attractive target for studies into the regulation of body weight. Reduced expression of *UCP3* decreases energy expenditure and increased expression of *UCP3* mRNA in muscle is related to an increase in the metabolic rate and to a lower body mass index (BMI)[9,10]. Therefore, *UCP3* may be involved in obesity, given the role of *UCP3* in the regulation of energy and lipid metabolism. Genetic variants of *UCP3* have been identified, and specifically polymorphisms of 55C/T may impact type 2 diabetes mellitus (T2DM), obesity and weight gain[11-13]. This study confirmed the hypothesis that polymorphisms of the *UCP3* are associated with the occurrence of NAFLD. These variations could be useful for the diagnosis and/or prognosis of NAFLD, although the functional significance of *UCP3* polymorphisms is not clear.

MATERIALS AND METHODS

Subjects

A total of 250 NAFLD children and 200 healthy individuals (controls), aged between 6 and 16 years were enrolled in this study. NAFLD children (147 males and 103 females) were referred to our endocrinology department from January 2006 to September 2011; NAFLD was defined according to the revised definition and treatment guidelines for NAFLD by the Chinese Hepatology Association in February 2006[14,15], and was diagnosed by means of a protocol using clinical, laboratory and ultrasound examinations in combination. In this study, NAFLD was diagnosed as a diffusely echogenic change on liver B-ultrasonography (fatty infiltration in liver), with or without elevated serum aminotransferase levels, and other factors which can cause liver fatty infiltration or aminotransferase elevation, such as infectious hepatitis (hepatitis B and C, Epstein-Barr virus infection), drug-induced hepatitis, and some metabolic diseases were excluded. None of the subjects had a history of alcohol consumption. Blood samples (n = 200) were also obtained from healthy individuals, who served as controls (109 males and 91 females) in 2011 from the Department of Child Health Care, The Affiliated Yuying Children's Hospital of Wenzhou Medical University and Ningbo Women and Children's Hospital. The protocol was approved by the Medical Ethics Committee of The Children's Hospital of Zhejiang University School of Medicine. Written informed consent were obtained from parents (or guardians) and children (where appropriate).

Laboratory assessment

The weight and height of the subjects were measured with a calibrated scale after removing shoes and heavy clothing, if any. BMI is calculated by taking the ratio of weight in kilogram and the square of height in meter. Waist was measured at the midpoint between the lower border of the rib cage and the iliac crest. Hip circumference was determined at the widest circle of the bottom. Venous blood samples were obtained from the subjects after an overnight fasting (12 h) for the measurement of fasting blood glucose (FBG), fasting insulin (FIN), total triglyceride (TG), total cholesterol (TCHO), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C), alanine transaminase (ALT) and aspartate aminotransferase (AST). All laboratory biochemical parameters were measured in a conventional automated analyzer.

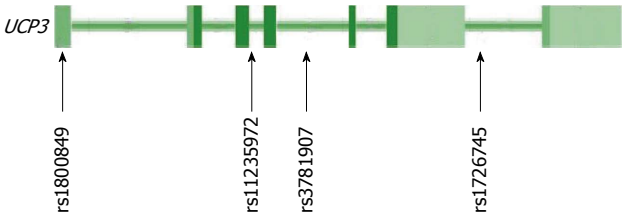

Figure 1 Locus of the human *UCP3* gene in 11q13. The uncoupling protein 3 (*UCP3*) gene consists of seven exons separated by six introns. Boxes indicate exons, while lines indicate introns and intergenic regions. Mark the polymorphism locations.

Liver ultrasound examination

Sagittal hepatic sections that encompassed longitudinal images of the right liver lobe and the ipsilateral kidney were obtained. Liver-kidney contrast with two other well-known ultrasonographic findings of fatty liver, vascular blurring and deep attenuate, enabled us to grade fatty change semi-quantitatively[15]. Ultrasound examination was carried out and blinded to laboratory values on the same equipment (GE, LOGIC 500), using a convex 3.5-5.0 MHz probe. NAFLD and healthy individuals underwent liver ultrasound examination.

DNA preparation and single nucleotide polymorphism genotyping

Using information on single nucleotide polymorphism (SNP) allelic frequencies from the website of the National Center for Biotechnology Information (NCBI) and the SNP browser software 3.0 (Applied Biosystems, Branchburg, NJ, United States), SNPs on the human *UCP3* gene with minor allele frequencies > 30% were selected. SNPs with relatively high minor allele frequencies have been shown to be very useful as genetic markers for genetic association studies. We selected four non-synonymous SNPs in the *UCP3* gene: polymorphisms of rs1726745, rs3781907, rs11235972, and rs1800849 (Figure 1). Genomic DNA was extracted from blood samples collected from each subject. Polymorphisms were genotyped using an automated platform MassARRAY (Sequenom, San Diego, CA). Polymerase chain reaction for the DNA sequence containing the target SNP was performed. The products were extended one base in SNP sites using the SNP specific primer. The products were applied into the MassARRAY SpectroCHIP array and crystallized with matrix in the chip. The crystal containing chip was moved to the mass spectrometer vacuum tube and excited using an instantaneous nanosecond (10^{-9} s) laser. The molecular of matrix absorb the radiation energy, which lead to energy accumulation causing crystal matrix sublimation, DNA molecule desorption and transformation to metastable ions.

Statistical analysis

Quantitative data with normal distribution were presented as mean ± SD. Categorical variables were expressed as a percentage and examined using the χ^2 test and Fisher's tests. Hardy-weinberg test was performed to calculate allelic frequencies using the χ^2 test. Multivariate logistic regression analysis by using stepwise selection was constructed to determine which of the potential risk factors of NAFLD were. Given the BMI, HOMA-IR, ALT, TCHO, rs1726745, rs3781907, rs11235972, and rs1800849 risk factors relative to the potential number of variables in our model, only those variables that had the highest possibility for independent prediction of outcome in our logistic regression were included. Multivariate logistic regression analysis was performed to estimate the OR and 95%CI for the potential risk factors of NAFLD. The statistical significance of means was estimated by independent *t* test. HOMA-IR = fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5.A *P* value of < 0.05 was regarded as statistical significant. All data analysis was done using the SPSS for windows (version 13.0; SPSS, Inc., Chicago, IL, United States). Haploview software (Cambridge, MA, United States) was used to screen tag SNPs.

Table 1 Demographic and biochemical features of patients with nonalcoholic fatty liver disease and normal controls

	NAFLD (n = 250)	Controls (n = 200)	*P* value
Gender (M/F)	147/103	109/91	0.36
Age (yr)	10.78 ± 2.07	10.63 ± 2.22	0.47
Body height (cm)	148.28 ± 11.77	141.22 ± 12.91	0.00
Body weight (kg)	62.82 ± 15.17	34.72 ± 10.28	0.00
BMI (kg/m^2)	28.13 ± 3.50	17.05 ± 2.16	0.00
SBP (mmHg)	114.02 ± 11.55	91.61 ± 9.68	0.00
DBP (mmHg)	68.57 ± 8.63	66.43 ± 7.67	0.01
Waist (cm)	89.84 ± 9.74	58.07 ± 7.04	0.00
Hip (cm)	93.85 ± 8.66	70.09 ± 7.85	0.00
WHR	0.95 ± 0.06	0.83 ± 0.05	0.00
FBG (mmol/L)	4.99 ± 0.41	4.91 ± 0.38	0.04
TCHO (mmol/L)	4.40 ± 0.89	4.14 ± 0.66	0.00
HDLc (mmol/L)	1.33 ± 0.51	1.52 ± 0.30	0.00
LDLc (mmol/L)	2.53 ± 0.72	2.19 ± 0.56	0.00
TG (mmol/L)	1.41 ± 0.84	0.80 ± 0.31	0.00
ALT (mmol/L)	72.70 ± 70.16	17.14 ± 10.48	0.00
AST (mmol/L)	47.26 ± 36.61	25.22 ± 6.53	0.00
FIN (mIU/L)	20.49 ± 17.25	6.98 ± 3.59	0.00
HOMA-IR	4.57 ± 3.92	1.52 ± 0.78	0.00

Data are expressed as absolute mean ± SD. Analysis was conducted using χ^2 test and *t* test. NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic pressure; WHR: Waist-to-hip ratio; FBG: Fasting blood glucose; TCHO: Total cholesterol; HDLc: High-density lipoprotein-cholesterol; LDLc: Low-density lipoprotein-cholesterol; TG: Total triglyceride; ALT: Alanine transaminase; AST: Aspartate aminotransferase; FIN: Fasting insulin; HOMA-IR: Homeostasis model assessment-estimated insulin resistance; M: Male; F: Female.

RESULTS

The clinical features of the NAFLD and control groups are shown in Table 1. There was no significant difference in age and gender (P > 0.05). NAFLD patients showed most of the risk factors for the MetS: elevated BMI, waist-to-height ratio (WHR), BP, FBG, HOMA-IR, TG, TCHO and LDL, while decreased HDL level compared to control group.

Table 2 The genotypic distributions of the four loci in the *UCP3* gene *n* (%)

	n	Genotypes			*P* value	Allele Frequency		*P* value
		Homozygous wild-type	Homozygous mutant-type	Heterozygous mutant-type		Mutant-type	Wild-type	
rs1726745		GG	AA	AG		A	G	
NAFLD	249	40 (16.1)	102 (41.0)	107 (43.0)	0.59	311 (62.4)	187 (37.6)	0.29
Controls	200	37 (18.5)	73 (36.5)	90 (45.0)		236 (59.0)	164 (41.0)	
rs3781907		TT	CC	CT		C	T	
NAFLD	239	87 (36.4)	42 (17.6)	110 (46.0)	0.23	194 (40.6)	284 (59.4)	0.19
Controls	198	57 (28.8)	37 (18.7)	104 (52.5)		178 (44.9)	218 (55.0)	
rs11235972		GG	AA	AG		A	G	
NAFLD	236	130 (55.1)	26 (11.0)	80 (33.9)	0.03	132 (28.0)	340 (72.0)	0.28
Controls	198	90 (45.5)	16 (8.0)	92 (46.5)		124 (31.3)	272 (68.7)	
rs1800849		CC	TT	TC		T	C	
NAFLD	249	133 (53.4)	25 (10.0)	91 (36.5)	0.14	141 (28.3)	357 (71.7)	0.54
Controls	199	92 (46.2)	16 (8.0)	91 (45.7)		123 (30.9)	275 (69.1)	

NAFLD: Nonalcoholic fatty liver disease.

Table 3 Multivariate regression analysis for risk factors of nonalcoholic fatty liver disease (*n* = 449)

	B	SE	Wald	*df*	Sig	Exp (B)	95%CI for EXP (B)	
							Lower	Upper
BMI	1.98	0.77	6.67	1	0.01	7.24	1.61	32.53
HOMA-IR	1.10	0.47	5.54	1	0.02	3.01	1.20	7.55
ALT	0.05	0.06	0.54	1	0.46	1.05	0.93	1.19
TCHO	-0.64	0.88	0.53	1	0.47	0.53	0.10	2.94
rs1800849	-7.28	40192.97	0	1	1	0	0	-
rs11235972	5.15	40192.97	0	1	1	172.72	0	-
rs3781907	1.09	2.01	0.30	1	0.59	2.98	0.06	151.89
rs1726745	-0.67	4.42	0.02	1	0.88	0.51	0	2933.6

TCHO: Total cholesterol; ALT: Alanine transaminase; HOMA-IR: Homeostasis model assessment-estimated insulin resistance; BMI: Body mass index.

The distributions of the four SNPs (rs1726745, rs3781907, rs11235972 and rs1800849) obeyed the Hardy-Weinberg equilibrium in all subjects. The genotypic distributions of the four loci in the *UCP3* gene are shown in Figure 1 and Table 2. The genotype distributions of rs11235972 in the NAFLD group differed significantly from that in the control group.

When all variables were put into multivariate logistic regression analysis, higher BMI and HOMA-IR were risk factors for the development of NAFLD (Table 3). The clinical features in subjects with or without rs1800849 variation are shown in Table 4. There were no significant differences in anthropometric and biomedical variables with or without rs11235972 variation in the NAFLD and in the control groups, respectively. We found body height between the CC (142.93 ± 13.08 cm) and CT+TT (139.38 ± 12.10 cm), waist circumference between the CC (58.76 ± 6.45 cm) and CT+TT (57.00 ± 5.59 cm) and hip circumference between the CC (71.28 ± 7.84 cm) and CT+TT (69.06 ± 7.75 cm) genotypes were significantly different in the control group with and without rs1800849 variation ($P < 0.05$).

When all genotypes of the four loci were entered into haplotype analysis using Haploview, only two haplotypes' alleles *i.e.*, GC and AT frequencies were accepted for assessment between the NAFLD and control groups. However, this did not reach the significance as an independent risk factor for NAFLD (Table 4 and Figure 2).

DISCUSSION

Previous reports have demonstrated that the prevalence of NAFLD increased 10%-80% in individuals with obesity, 35%-90% in individuals with T2DM, 30%-56% in individuals with hypertension, and 26%-58% in individuals with dyslipidemia[16-18]. The prevalence of the MetS among subjects with NAFLD is 17%-36%, depending on gender and criteria used[19]. In the present study, we found that an increased risk of NAFLD was significantly associated with BMI, WHR, BP, FBG, HOMA-IR, TG, TCHO and LDL, and decreased HDL level. In agreement with these studies, Iacobellis *et al*[20] reported that a BMI evaluation may be useful in identifying those children at higher risk for disease progression. In the present study, children with NAFLD had an elevated WHR, a surrogate marker for visceral fat. Visceral fat is closely correlated with hepatic TG content, elevated ALT, liver inflammation, and fibrosis[21-23]. Central obesity is a better measure of predisposition to insulin resistance, and is more closely associated with NAFLD.

UCP3 is considered to be associated with obesity, given the role for *UCP3* in the regulation of energy and

Table 4 Demographic and biochemical features of subjects with and without rs1800849 variation (mean ± SD)

	Control			NAFLD		
	CC (n = 92)	CT+TT (n = 107)	P value	CC (n = 133)	CT+TT (n = 116)	P value
Gender (M/F)	48/44	60/47	0.582	103/30	90/26	0.979
Body height (cm)	142.93 ± 13.08	139.38 ± 12.10	0.048	148.82 ± 12.50	147.83 ± 10.82	0.510
Body weight (kg)	35.65 ± 9.69	33.29 ± 8.51	0.070	64.42 ± 16.86	61.14 ± 12.74	0.083
BMI (kg/m^2)	17.11 ± 2.00	16.86 ± 1.80	0.358	28.45 ± 3.75	27.79 ± 3.17	0.141
SBP (mmHg)	91.59 ± 8.89	91.29 ± 9.80	0.824	113.32 ± 11.74	114.84 ± 11.36	0.301
DBP (mmHg)	66.71 ± 7.11	66.13 ± 8.16	0.595	68.27 ± 9.13	68.91 ± 8.08	0.561
Waist (cm)	58.76 ± 6.45	57.00 ± 5.59	0.040	90.78 ± 10.58	88.87 ± 8.57	0.123
Hip (cm)	71.28 ± 7.84	69.06 ± 7.75	0.047	94.31 ± 9.51	93.47 ± 7.64	0.483
WHR	0.83 ± 0.05	0.83 ± 0.04	0.833	0.95 ± 0.07	0.95 ± 0.05	0.559
FBG (mmol/L)	4.91 ± 0.35	4.92 ± 0.40	0.888	5.02 ± 0.44	4.96 ± 0.36	0.245
TG (mmol/L)	4.11 ± 0.65	4.17 ± 0.67	0.519	4.32 ± 0.82	4.48 ± 0.96	0.165
HDL (mmol/L)	1.48 ± 0.29	1.55 ± 0.30	0.128	1.33 ± 0.58	1.35 ± 0.40	0.743
LDL (mmol/L)	2.18 ± 0.52	2.19 ± 0.58	0.950	2.50 ± 0.70	2.56 ± 0.75	0.457
ALT (mmol/L)	16.85 ± 6.44	16.48 ± 8.92	0.740	54.37 ± 2.07	51.43 ± 2.28	0.574
AST (mmol/L)	23.90 ± 5.54	26.07 ± 6.56	0.013	47.53 ± 36.24	46.91 ± 37.35	0.896
FINS	7.20 ± 3.34	6.62 ± 3.35	0.226	17.13 ± 2.11	14.86 ± 2.12	0.140
HOMA-IR	1.58 ± 0.75	1.45 ± 0.74	0.231	3.80 ± 2.14	3.27 ± 2.12	0.117

Analysis was conducted using t test. Statistically significant differences between groups are shown in bold. NAFLD: Nonalcoholic fatty liver disease; M: Male; F: Female; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic pressure; WHR: Waist-to-hip ratio; FBG: Fasting blood glucose; HDL: High-density lipoprotein-cholesterol; LDL: Low-density lipoprotein-cholesterol; TG: Total triglyceride; ALT: Alanine transaminase; AST: Aspartate amino-transferase; FIN: Fasting insulin; HOMA-IR: Homeostasis model assessment-estimated insulin resistance.

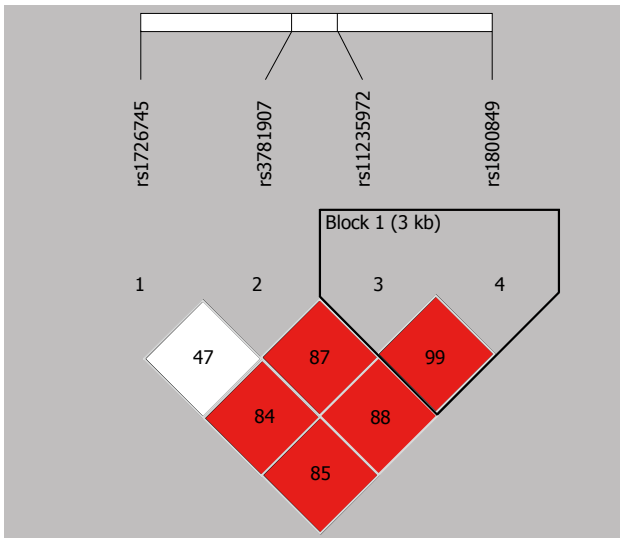

Figure 2 Linkage Disequilibrium mapping around the uncoupling protein 3 between nonalcoholic fatty liver disease and control groups.

Table 5 Haplotype frequencies of rs1800849, rs11235972, rs3781907, rs1726745

Block	Frequencies		χ^2	P value
	NAFLD	Control		
GC	0.712	0.682	0.901	0.343
AT	0.282	0.307	0.702	0.402

NAFLD: Nonalcoholic fatty liver disease.

lipid metabolism. However, the effect of genetic polymorphisms in *UCP3* on the pathogenesis of NAFLD has not been clearly documented. In our study, the GG genotype distribution of rs11235972 in the NAFLD group differed significantly from the control group. The results from the literature are controversial. Yoon *et al*[24] genotyped 6 polymorphisms of *UCP3* among overweight female subjects (n = 458), and genetic effects on BMI and changes after a very low calorie diet were examined. They found that several polymorphisms in the *UCP2-3* gene cluster showed associations with changes in BMI and fat mass. Hamada *et al*[25] determined whether the *UCP3*-55 C/T SNP was associated with obesity according to the criteria for Japanese (BMI ⩾ 25 kg/m^2) and serum HDL-C levels in the general population. Subjects with the T/T genotype had significantly higher HDL-C levels than those with the C/C genotype or C/T genotype. Furthermore, subjects with the T/T genotype had a significantly lower BMI than those with the C/C genotype[25]. Salopuro *et al*[26] found that the *UCP3* gene variant rs3781907 was associated with increased serum TCHO and LDL cholesterol levels. The rs1726745, rs11235972 and rs1800849 variants in the *UCP3* gene are associated with serum total and LDL-cholesterol at baseline. However, de Luis *et al*[27] did not demonstrate an association between the -55CT polymorphism of the *UCP3* gene and fat distribution in obese patients. There might be true variability in the association among different populations, particularly different ethnic groups.

A common promoter polymorphism has also been identified in the *UCP3* gene (rs1800849), a rare allele associated with obesity in a recessive manner in several studies[10-12]. Moreover, the rs1800849 allele is associated with a higher WHR[28], but no association between rs1800849 and WHR existed in the current study. We showed a significant difference in height, and waist and hip circumference between the CC (mutant type group) and CT+TT group with and without rs1800849 variation (Table 5).

One limitations in this study was that we used abdominal ultrasonography to diagnose NAFLD, although validation ultrasonography has a sensitivity of 91.7% and a specificity of 100%[29]. The diagnosis of NAFLD was based on ultrasound and was not confirmed by liver biopsy due to the invasive procedure usually not initially performed and ethical considerations. Thus, surrogate markers are commonly used, such as transaminases and imaging techniques. Computed tomography is more specific but is not used for screening of fatty liver in obese children. Magnetic tomography is more useful in adults and not appropriate for children due to ionizing radiation. Magnetic resonance imaging and 1H-MRS have the greatest accuracy to determine hepatic fat content, but are rarely used due to high costs. A higher prevalence of the rs11235972 GG genotype was noted in the NAFLD group compared with the control group; no differences were observed for the other SNPs. BMI and HOMA-IR increased the risk of NAFLD. Moreover, no increased risk for developing NAFLD was found to be associated with the rs1800849 variant based on multivariate analysis. A significant difference in height, and waist and hip circumference between the CC and CT+TT group with and without rs1800849 variation was demonstrated.

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COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathologic condition characterized by abnormal lipid deposition in hepatocytes in the absence of excess alcohol intake. There are few population-based prevalence studies of pediatric NAFLD. Elucidation of genetic factors that predispose an individual to NAFLD may lead to development of non-invasive biomarkers for the early diagnosis of NAFLD.

Research frontiers

Uncoupling protein 3 (*UCP3*) is considered to be associated with obesity, given the role for *UCP3* in the regulation of energy and lipid metabolism. However, the effect of genetic polymorphisms in *UCP3* on the pathogenesis of NAFLD has not been clearly documented.

Innovations and breakthroughs

Theoretically, many variations in candidate genes related to MetS may contribute to the pathogenesis of NAFLD. This is the first study to report that there significant difference of body height, waist and hip circumference between CC (mutant type group) and CT+TT group with and without rs1800849 variation were found.

Applications

This study confirmed the hypothesis that polymorphisms of the *UCP3* are associated with the occurrence of NAFLD. These variations could be useful for the diagnosis and/or prognosis of NAFLD.

Terminology

UCP3 gene is located on chromosome 11q13. *UCP3* is a mitochondrial anion carrier protein with a highly selective expression in skeletal muscle, a major site of thermogenesis in humans, which makes an attractive target for studies into the regulation of body weight.

Peer review

The authors examined the polymorphisms of the *UCP3* gene associated with the occurrence of NAFLD. Higher rs11235972 GG genotype prevalence has been observed in the NAFLD group compared with the control group. There significant difference of body height, waist and hip circumference between CC (mutant type group) and CT+TT group with and without rs1800849 variation were found. This is an interesting manuscript about NAFLD in children and *UCP3* polymorphisms. The manuscript has adequate methodology and is good written.

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BRIEF ARTICLE

Influence of chronic HBV infection on superimposed acute hepatitis E

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Abstract

AIM: To investigate the influence of chronic hepatitis B virus (HBV) infection [based on the status of hepatitis B e antigen (HBeAg), HBV DNA, and cirrhosis] on superimposed acute hepatitis E.

METHODS: A total of 294 patients were recruited from the Department of Infectious Diseases of the Third Affiliated Hospital, Sun Yat-sen University, from January 2003 to January 2012. The patients were classified into two groups: an HBV + hepatitis E virus (HEV) group (a group with chronic HBV infection that was superinfected with acute hepatitis E, n = 118) and an HEV group (a group with acute hepatitis E, n = 176). We retrospectively analyzed and compared the clinical features of the two groups. Statistical analyses were performed using the χ^2 test or Fisher's exact test for categorical variables and the Student's t test for continuous variables. A P value < 0.05 was considered statistically significant.

RESULTS: The peak values of prothrombin time, serum total bilirubin, and Model for End-Stage Liver Disease scores were significantly higher in the HBV + HEV group. More patients in the HBV + HEV group had complications (39.8% *vs* 16.5%, P = 0.000) and developed liver failure (35.6% *vs* 8.5%, P = 0.000). Additionally, the mortality of the HBV + HEV group was significantly higher (20.3% *vs* 7.4%, P = 0.002). Further analysis of the HBV + HEV group showed that there were no significant differences in complication occurrence, liver failure incidence, or mortality between patients with different HBeAg and HBV DNA statuses. However, in patients with underlying cirrhosis, complication occurrence and liver failure incidence significantly increased. In total, 12.7% of the patients in the HBV + HEV group received anti-HBV treatment, but this therapy failed to reduce mortality in patients who developed liver failure.

CONCLUSION: The presence of underlying cirrhosis in chronic HBV infection results in more severe clinical outcomes with superimposed acute hepatitis E. Anti-HBV treatment cannot improve the prognosis of liver failure caused by HBV-HEV superinfection.

Key words: Chronic hepatitis B virus infection; Acute hepatitis E; Superinfection; Clinical profile; Anti-hepatitis B virus treatment

Core tip: Previous studies have shown that chronic hepatitis B virus (HBV) infection has a negative impact on superimposed acute hepatitis E. However, it remains unknown whether the disease severity of acute hepatitis E correlates with the underlying HBV replication status or with liver histological lesions. Our study

showed that the disease severity of acute hepatitis E correlated not with the HBV replication status (based on the status of hepatitis B e antigen and HBV DNA), but rather with the presence of underlying cirrhosis. This finding raised the question of whether anti-HBV treatment improves the outcome of liver failure caused by HBV-hepatitis E virus superinfection. We found that anti-HBV treatment could not improve the prognosis of such liver failure.

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INTRODUCTION

Infection by hepatitis B virus (HBV) is a serious public health problem worldwide. Two billion people worldwide have been infected with HBV, including more than 240 million cases of chronic infection[1,2]. During the chronic course of HBV infection, there is a chance that patients may be sporadically superinfected with other viruses, such as hepatitis E virus (HEV). HEV is mainly endemic in tropical and subtropical developing countries, including China. Studies of serum epidemiology in China showed that HEV superinfection in patients with chronic hepatitis B is present in 17.6% of these patients[3].

HEV generally causes an acute, self-limiting illness, followed by a complete recovery. Recent studies have shown that HEV can result in severe disease in patients with underlying chronic HBV infection and even liver failure[4-7]. In the chronic course of HBV infection, there are different statuses of hepatitis B e antigen (HBeAg) and HBV DNA, and certain patients have a higher probability of developing cirrhosis. No previous studies are available regarding whether these different chronic statuses have different influences on the superinfection of HBV and HEV.

Liver failure related to HBV activation remains a rapidly progressive and frequently fatal condition. Traditional treatment is generally supportive. International HBV treatment guidelines recommend initiating nucleos(t)ide analogs as early as possible in this patient population[8,9]. Studies on the efficacy of nucleoside analogs have been emerging in recent years. Recent studies have shown that anti-HBV treatment could improve the outcome of this patient population[10-13]. Superinfection with HEV is another common cause of liver failure in patients with chronic HBV infection, accounting for 20% of cases in regions endemic for HEV[4]. Importantly, such liver failure caused by HBV and HEV results in high mortality rates. However, there are still no data on anti-HBV treatment for liver failure caused by the superinfection of HBV and HEV, as previous studies did not consider patients with superinfection.

The aim of our study was to investigate the impact of chronic HBV infection on superimposed acute hepatitis E, particularly the influence of the status of HBeAg, HBV DNA, and cirrhosis on disease severity. Furthermore, we evaluated the effect of anti-HBV treatment on HBV-HEV superinfection. The use of a single liver function index is limited in assessing liver function, but the Model for End-Stage Liver Disease (MELD) score[14], which combines multiple indices, can play a useful role in this assessment. The MELD score system has been used extensively for the allocation of donor livers worldwide[15] and has been validated for use in chronic hepatitis B (CHB)[16]. Thus, the MELD score was applied for a comprehensive analysis of liver function.

MATERIALS AND METHODS

Patients

This work was approved by the local ethics committee of our university. A total of 294 patients were recruited from the Department of Infectious Diseases of the Third Affiliated Hospital, Sun Yat-sen University, from January 2003 to January 2012. Among these patients, 118 were diagnosed with acute hepatitis E and chronic HBV superinfection (HBV + HEV group), and 176 patients were diagnosed with acute hepatitis E alone (HEV group). Acute hepatitis E was diagnosed when patients were hospitalized with typical symptoms of acute viral hepatitis and the presence of anti-HEV serum IgM and IgG. The presence of HBsAg and the absence of anti-HBc IgM established a diagnosis of chronic HBV infection. The diagnosis of liver failure was based on the Guidelines for Diagnosis of Liver Failure (2006)[17] and included the presence of two or more of the following: an international normalized ratio (INR) $\geq$ 1.5, serum total bilirubin (TBil) > 10 times the upper limit of normal, ascites, hepatic encephalopathy, decreased liver size, or hepatorenal syndrome. The complications that were observed were ascites, peritonitis, hepatic encephalopathy, gastrointestinal bleeding, and hepatorenal syndrome.

Detection

Anti-HEV serum IgM and IgG were detected with an enzyme-linked immunosorbent assay (Genelabs Technologies, Singapore). HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb were detected with an automatic rapid immunoassay system (AxSYM; Abbott, United States). HBV DNA levels were determined by real-time polymerase chain reactions using commercial diagnostic kits (Da-an GeneCo., Guangzhou, China) with a lower detection limit of 500 copies/mL. Liver function tests were performed using an automatic biochemical analyzer (AU 640; Olympus, Japan). In this study, prothrombin time (PT)-INR (PT/reference PT) = international sensitivity index. The PT was measured using the detection reagent STA-Neoplastine(r) CI PLUS with an automatic coagulometer (STA-R) (Diagnostica Stago, France). A diagnosis of underlying cirrhosis was made based on clinical,

Table 1 Demographic and clinical characteristics of the hepatitis B virus + hepatitis E virus group and hepatitis E virus group

	HBV + HEV group (n = 118)	HEV group (n = 176)	P values
Age (yr)	44.5 ± 13.8	54.1 ± 15.8	0.000[1]
Sex			0.000[1]
Male	109 (92.4)	140 (79.5)	
Female	9 (7.6)	36 (20.5)	
ALT (U/L)	266.0 ± 227.3	262.9 ± 212.9	0.905
AST (U/L)	228.9 ± 207.1	228.4 ± 213.1	0.986
PT (s)	22.1 ± 11.3	16.8 ± 7.9	0.000[1]
PTA (%)	57.3 ± 26.6	77.2 ± 24.9	0.000[1]
TBil (μmol/L)	334.7 ± 228.0	277.5 ± 217.4	0.031[1]
MELD score	20.0 ± 9.7	15.1 ± 8.6	0.000[1]
Complications	47 (39.8)	29 (16.5)	0.000[1]
Liver failure	42 (35.6)	15 (8.5)	0.000[1]
Death	24 (20.3)	13 (7.4)	0.002[1]

[1]Denotes significant P value. Data are expressed as absolute n (%) or mean ± SD. HBV: Hepatitis B virus; HEV: Hepatitis E virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin time; PTA: Prothrombin activity; TBil: Serum total bilirubin; MELD: Model for End-Stage Liver Disease.

biochemical, and ultrasonographic findings. Sample collection, transportation, preservation, and processing were performed according to the manufacturer's instructions.

Calculation of MELD scores

MELD score = 3.8 × $\log_e$ [serum bilirubin (μmol/L) × 0.058] + 11.2 × $\log_e$ (PT-INR) + 9.6 × $\log_e$ [serum Cr (μmol/L) × 0.011] + 6.4 × (0 or 1) (cholestatic or alcoholic cirrhosis: 0; other liver diseases: 1)[14].

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, United States). The χ^2 test or Fisher's exact test were used for categorical variables, and the Student's t test was used for continuous variables. Continuous variables are expressed as the mean ± SD, and categorical variables are expressed as the percentage (number). P values < 0.05 were considered statistically significant.

RESULTS

Demographic characteristics

The demographic characteristics of the 294 patients are shown in Table 1. The males outnumbered the females in both groups. The mean ages at admission were 44.5 and 54.1 years in the HBV + HEV and HEV groups, respectively.

Laboratory findings

Liver function tests were performed at admission and regularly after admission. We compared the most severe laboratory abnormalities in the biochemical profile between the two groups (Table 1). The mean peak values of PT (22.1 s *vs* 16.8 s, P = 0.000) and TBil (334.7 μmol/L *vs* 277.5 μmol/L, P = 0.031), as well as the mean MELD score (20.0 *vs* 15.1, P = 0.000), were significantly higher in the HBV + HEV group compared with the HEV group. In contrast, the mean values of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) did not differ significantly between the two groups.

Clinical outcomes

As shown in Table 1, the incidences of complications, liver failure, and death were compared between the two groups with respect to clinical outcomes. Complications were noted in 39.8% (47/118) and 16.5% (29/176) of the patients in the HBV + HEV and HEV groups, respectively, and the occurrence of complications in the HBV + HEV group was significantly higher than that in the HEV group (P = 0.000). The incidence of liver failure was significantly higher among patients with superinfection than among patients with acute hepatitis E alone (35.6% *vs* 8.5%, P = 0.000). The mortality rates were also significantly different between the two groups (20.3% in the HBV + HEV group and 7.4% in the HEV group).

Influence of chronic status of HBV infection on acute hepatitis E

To evaluate the influence of chronic HBV infection (based on the status of HBeAg, HBV DNA, and cirrhosis), we performed further analysis of the HBV + HEV group (Table 2). Of the 118 patients in the HBV + HEV group, 16.9% (20/118) were HBeAg-positive, 55.1% (65/118) were HBV DNA-positive, and 14.4% (17/118) had underlying cirrhosis. The occurrence of complications, liver failure, and death did not differ significantly between the HBeAg (+/-) and HBV DNA (+/-) subgroups. Patients with underlying cirrhosis had a significantly higher incidence of complications and liver failure. The mortality rate was 23.5% in the cirrhosis subgroup and 19.8% in the non-cirrhosis subgroup, which was not a significant difference.

Anti-HBV treatment in the HBV + HEV group

Of the 118 patients in the HBV + HEV group, only 15 (12.7%) patients took oral anti-HBV agents. Three of these patients received lamivudine, and the other 12 patients received entecavir. In the HBV + HEV group, 42 patients developed liver failure, and 28.6% received anti-HBV treatment. Only 3.9% of patients without liver failure received anti-HBV treatment.

The mean mortality rates among the 42 patients with liver failure were 66.7% and 53.3% for patients who were and were not receiving anti-HBV treatment, respectively, which were not significantly different (Table 3).

DISCUSSION

Due to the high prevalence of both HBV and HEV infection and the lack of cross-immunity between the two viruses, HBV-HEV superinfection is common[3,18].

In our study, patients with acute hepatitis E superimposed on chronic HBV infection had higher peak laboratory abnormalities and poorer outcomes. There was also

Table 2 Influence of chronic hepatitis B virus infection on acute hepatitis E *n* (%)

	HBV + HEV group								
	Status of HBeAg			Status of HBV DNA			Status of cirrhosis		
	+ (*n* = 20)	- (*n* = 98)	*P* value	+ (*n* = 65)	- (*n* = 53)	*P* value	+ (*n* = 17)	- (*n* = 101)	*P* value
MELD score	17.0 ± 7.0	20.6 ± 10.1	0.131	21.5 ± 10.6	18.3 ± 8.3	0.074	24.7 ± 12.2	19.2 ± 9.1	0.03[1]
Liver failure	5 (25)	37 (37.8)	0.317	25 (38.5)	17 (32.1)	0.563	11 (64.7)	31 (30.7)	0.01[1]
Complications	7 (35)	40 (40.8)	0.803	25 (38.5)	22 (41.5)	0.850	16 (94.1)	31 (30.7)	0.00[1]
Death	2 (10)	22 (22.4)	0.359	14 (21.5)	10 (19.9)	0.820	4 (23.5)	20 (19.8)	0.748

[1]Denotes significant *P* value. HBV: Hepatitis B virus; HEV: Hepatitis E virus; HBeAg: Hepatitis B e antigen; MELD: Model for End-Stage Liver Disease.

Table 3 Anti-hepatitis B virus treatment in patients with liver failure in the hepatitis B virus + hepatitis E virus group

	Patients receiving anti-HBV treatment (*n* = 12)	Patients not receiving anti-HBV treatment (*n* = 30)	*P* value
HBV DNA (log_{10} copies/mL)	4.99e7 ± 9.82e7	2.35e8 ± 9.20e8	0.495
MELD score	33.2 ± 9.4	28.3 ± 9.2	0.129
Mortality	8 (66.7)	53.3 (16/30)	0.506

Data are expressed as absolute *n* (%) or mean ± SD. HBV: Hepatitis B virus; HEV: Hepatitis E virus; MELD: Model for End-Stage Liver Disease.

a higher prevalence of liver failure among those patients. The present study confirmed the previous finding that acute HEV infection can cause severe liver injury in patients with chronic HBV infection[4-7]. This result indicates that chronic HBV infection has a negative impact on the clinical features of acute hepatitis E. However, it remains unknown whether the disease severity of acute hepatitis E correlates with the underlying HBV replication status or liver histological lesions. Our further analysis of the superinfection group showed that the disease severity of superimposed acute hepatitis E correlated not with the HBV replication status (based on the status of HBeAg and HBV DNA), but rather with the presence of underlying liver histological lesions (liver cirrhosis).

It has long been suggested that patients with chronic HBV infection are immunologically different from people without HBV infection. For instance, patients with chronic HBV infection have been reported to have impaired cell-mediated immunity[19-22], decreased peripheral-blood T cell numbers[23,24], impaired interferon production[25,26], and imbalanced cytokine levels[27,28] and may have other currently unrecognized differences. The severity of acute viral hepatitis has been suggested to be dependent on host immune factors rather than on the direct toxicity of the virus. Thus, with impaired and imbalanced immunity in chronic HBV infection, HEV may trigger an excessive immunological response and then induce severe damage in hepatocytes. Alternatively, hepatocyte impairment may accumulate during the chronic course of HBV infection. Thus, with preexisting liver lesions, especially due to cirrhosis, hepatocytes may be limited in their ability to regenerate. This limitation contributes to more severe liver injury in patients with acute hepatitis E superimposed on chronic HBV infection.

According to our data, most patients in the HBV + HEV group were HBeAg-negative, and nearly 50% were HBV DNA-negative. Further analysis showed that the disease severity of acute hepatitis E did not correlate with the status of HBeAg or HBV DNA. This finding indicates that in the superinfection of HBV and HEV, chronic HBV infection is inactive, and HEV is the main trigger factor for severe disease. Thus, the finding raises the question of whether anti-HBV treatment improves the outcome of HBV-HEV superinfection, which requires further investigation.

Acute exacerbation frequently occurs in the natural course of chronic HBV infection. In the case of acute exacerbation caused by spontaneous HBV activation, anti-HBV treatment can strongly suppress HBV replication, and most patients can recover. However, certain patients may develop liver failure, which is named HBV-related acute-on-chronic liver failure (HBV-ACLF). HBV-ACLF remains a rapidly progressive and frequently fatal condition for which mortality reaches 25% to 35%. International guidelines recommend initiating nucleos(t)ide analogs as early as possible in this patient population[8,9]. Recent studies have shown that anti-HBV treatment can improve the outcome of HBV-ACLF[10-13]. Superinfection with HEV is another common cause of liver failure in chronic HBV infection and is present in 20% of cases in regions endemic for HEV[4]. Importantly, such liver failure caused by HBV and HEV results in high rates of mortality. However, for the liver failure caused by the superinfection of HBV and HEV, there are still no data on anti-HBV treatment. In our study, we evaluated the results of anti-HBV treatment administration to the HBV + HEV group. Of the 76 patients without liver failure, only 3.9% took anti-HBV drugs, but the prognosis of this patient population was good. This finding indicates that it is not necessary to administer anti-HBV treatment as soon as possible to patients with HBV-HEV superinfection in mild disease. The necessity of anti-HBV treatment for HBV infection should be re-evaluated by monitoring the HBV DNA level and liver function tests after recovery from acute hepatitis E.

As shown by our data, up to 28.6% of patients received anti-HBV treatment once superinfection caused liver failure. The mortality rate among patients receiving anti-HBV treatment was 66.7%, which was not significantly different from the mortality rate of patients not receiving anti-HBV treatment. Thus, anti-HBV treatment

was unable to improve the outcome of the liver failure caused by HBV-HEV superinfection. As mentioned previously, HEV infection plays the most important role in the disease. The infection triggers strong immunological injury in hepatocytes, which results in liver failure. Anti-HBV treatment can inhibit HBV replication but cannot stop the strong immune activity, so the therapy cannot improve the outcome of this patient population.

In conclusion, our study indicates that acute hepatitis E is associated with more severe disease in patients with chronic HBV infection and that disease severity correlates with the underlying cirrhosis in chronic HBV infection. Anti-HBV treatment cannot improve the prognosis of liver failure caused by HBV-HEV superinfection. As HBV vaccination is being aggressively pursued worldwide, HEV vaccination should also be considered in endemic areas when a vaccine becomes available[29]. Additionally, preventive measures are important to the related morbidity and mortality, such as the consumption of boiled water and well-cooked food.

The main limitation of the present study is its retrospective nature. HEV-RNA levels were not regularly checked, so we are not convinced that there is no substantial contribution of HEV load to the disease severity of HBV-HEV superinfection. Additionally, underlying cirrhosis was mainly assessed by ultrasonography. Thus, it is possible that early-stage underlying cirrhosis was missed. Despite these limitations, our study is significant because the work provides preliminary support for not administering anti-HBV treatment in HBV-HEV superinfection, as chronic HBV replication status (based on the status of HBeAg and HBV DNA) does not determine the outcome of HBV-HEV superinfection.

COMMENTS

Background

Hepatitis B virus (HBV)-hepatitis E virus (HEV) superinfection is common. Recent studies have shown that HEV can result in severe disease in patients with underlying chronic HBV infection and even liver failure. However, whether the disease severity of acute hepatitis E correlates with the underlying HBV replication status (based on the status of HBeAg and HBV DNA) or liver histological lesions and whether anti-HBV treatment can improve the outcome of HBV-HEV superinfection are unknown.

Research frontiers

The present study investigated the influence of chronic HBV infection (based on the status of HBeAg, HBV DNA, and cirrhosis) on superimposed acute hepatitis E. Furthermore, the study evaluated the effect of anti-HBV treatment on HBV + HEV superinfection.

Innovations and breakthroughs

The disease severity of superimposed acute hepatitis E correlated not with the HBV replication status (based on the status of HBeAg and HBV DNA), but rather with the presence of underlying cirrhosis. Anti-HBV treatment did not improve the prognosis of liver failure caused by HBV-HEV superinfection.

Peer review

This study is of great interest and offers useful implications for the treatment of HBV-HEV superinfection.

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BRIEF ARTICLE

A phase Ⅱ study of paclitaxel and nedaplatin as front-line chemotherapy in Chinese patients with metastatic esophageal squamous cell carcinoma

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Abstract

AIM: To evaluate the efficacy and safety of paclitaxel-nedaplatin combination as a front-line regimen in Chinese patients with metastatic esophageal squamous cell carcinoma (ESCC).

METHODS: A two-center, open-label, single-arm phase Ⅱ study was designed. Thirty-nine patients were enrolled and included in the intention-to-treat analysis of efficacy and adverse events. Patients received 175 mg/m^2 of paclitaxel over a 3 h infusion on 1 d, followed by nedaplatin 80 mg/m^2 in a 1 h infusion on 2 d every 3 wk until the documented disease progression, unacceptable toxicity or patient's refusal.

RESULTS: Of the 36 patients assessable for efficacy, there were 2 patients (5.1%) with complete response and 16 patients (41.0%) with partial response, giving an overall response rate of 46.1%. The median progression-free survival and median overall survival for all patients were 7.1 mo (95%CI: 4.6-9.7) and 12.4 mo (95%CI: 9.5-15.3), respectively. Toxicities were moderate and manageable. Grade 3/4 toxicities included neutropenia (15.4%), nausea (10.3%), anemia (7.7%), thrombocytopenia (5.1%), vomiting (5.1%) and neutropenia fever (2.6%).

CONCLUSION: The combination of paclitaxel and nedaplatin is active and well tolerated as a first-line therapy for patients with metastatic ESCC.

Key words: Esophageal squamous cell cancer; Front-line chemotherapy; Paclitaxel; Nedaplatin

Core tip: Esophageal cancers are among the most aggressive tumors with a poor prognosis. Till now, there has been no standard chemotherapy regimen for advanced esophageal cancer. In this paper, we conducted a phase Ⅱ study on combination chemotherapy consisting of paclitaxel and nedaplatin in previously untreated patients with metastatic esophageal squamous cell carcinoma (ESCC). Our results demonstrated that the combination of two drugs is active and well tolerated as a first-line therapy for patients with recurrent or metastatic ESCC.

He YF, Ji CS, Hu B, Fan PS, Hu CL, Jiang FS, Chen J, Zhu L, Yao YW, Wang W. A phase Ⅱ study of paclitaxel and nedaplatin

as front-line chemotherapy in Chinese patients with metastatic esophageal squamous cell carcinoma. *World J Gastroenterol* 2013; 19(35): 5910-5916 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5910.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5910

INTRODUCTION

China accounts for about half of the world's esophageal cancer cases, about 250000 each year, and 85% of the total global incidence occurs in the developing world, according to the World Health Organization report. Overt and incurable metastatic disease is present at diagnosis in 50% of patients. Furthermore, even after curative surgery, local recurrences and/or distant metastases are detected in more than 50% of the patients within 5 years of follow-up[1]. The median survival of patients with metastatic esophageal carcinoma is only 3-8 mo[2]. Palliative chemotherapy may lead to distant tumor and symptom control. The effect of chemotherapy on survival is unclear for lack of large randomized trials. Up till now there has been no global standard regimen for the first-line treatment of advanced disease. Of the available regimens, the regimen containing 5-fluorouracil (5-FU) and cisplatin is widely used in China, with RR ranging from 15%-45%[3-5]. However, treatment with 5-FU and cisplatin can induce severe toxicity[6]. What's more, almost all patients have to be hospitalized for this treatment. Therefore, it is imperative to develop effective and well-tolerated chemotherapeutic agents for treatment.

Recently, paclitaxel, a natural product isolated from the bark of the yew tree Taxus brevifolia, has demonstrated some promising responses against digestive tract cancer. As a single agent, paclitaxel has been reported to achieve a response rate of 32% in esophageal cancer and gastroesophageal junction cancer[7]. Besides, several phase Ⅰ/Ⅱ studies have shown that paclitaxel-based regimens have significant activity in patients with locally advanced and metastatic esophageal cancer[8-12]. However, toxicity for combination therapy was significant and included severe myelosuppression, gastrointestinal (GI) and neurologic toxicity, and a significant rate of hospitalization for treatment-related complications. So it is urgent to seek new combination treatments that could achieve similar outcome and induce relatively minimal toxicities.

Nedaplatin cis-diammine-glycolate platinum (NDP) is a new platinum derivative, selected from a series of platinum analogues based on its pronounced preclinical antitumor activity against various solid tumors with lower nephrotoxicity[13]. Preclinical studies indicate that nedaplatin has an antitumor activity comparable to cisplatin[14-16] and has been shown experimentally to overcome cisplatin resistance in a cisplatin-resistant K562 cell line[14]. Clinically, single agent nedaplatin has shown a wide spectrum of antitumor activity, producing the favorable response rates in head and neck[17], esophagus[18], non-small cell lung[19,20], and cervical cancers[21]. These reports prompted us to use a new combination of nedaplatin and paclitaxel for patients with metastatic esophageal carcinoma, because these patients have poorer tolerance, and a less toxic treatment is desirable. The current phase Ⅱ study was conducted to evaluate the efficacy and safety of nedaplatin-paclitaxel combination as a front-line regimen in Chinese patients with metastatic esophageal squamous cell carcinoma.

MATERIALS AND METHODS

Study design

This was a two-center, open-label, single-arm phase Ⅱ study evaluating the efficacy and toxicities of nedaplatin and paclitaxel in patients with metastatic esophageal squamous cell carcinoma who had no previous treatment. The primary end point was response to treatment. Secondary end points were toxicity, progression-free survival (PFS) and overall survival (OS).

Eligibility criteria

Patients aged 18-75 years with measurable target lesion pathologically confirmed advanced or metastatic esophageal squamous cell carcinoma were eligible for the study. Prior chemotherapy for advanced disease was not permitted. However, neoadjuvant or concurrent chemotherapy was allowed, provided that the treatment was completed at least 6 mo before the start of the current study. Patients were required to have Eastern Cooperative Oncology Group (ECOG) performance status of 0-2, with a life expectancy $\geqslant$ 3 mo, an adequate bone marrow, liver and kidney function, as indicated by an absolute neutrophil count (ANC) $\geqslant$ 1500/μL, a platelet count $\geqslant$ 100000/μL, serum creatinine $\leqslant$ 2.0 mg/dL, serum bilirubin $\leqslant$ 1.5 mg/dL, and serum alanine aminotransferase $\leqslant$ 2.5 times higher than the upper limit of the normal (except in those cases with liver involvement when a value $\leqslant$ 5 times the upper limit of the normal was accepted). All patients were given written informed consents to participate in this study, which was also approved by the Ethics Committee of two centers.

Exclusion criteria

Patients with evidence of central nervous system metastases, an inability to take oral medication were excluded. Gastroesophageal junction tumors were excluded from the study. Exclusion criteria also included pathologically confirmed adenocarcinoma, prior malignancies (other than non-melanoma skin cancer or *in situ* cervical cancer) within the previous 5 years, and uncontrolled infection or severe comorbidity such as myocardial infarction within 6 mo or symptomatic heart diseases. Pregnant or lactating women were excluded from the study; women with childbearing potential were required to agree to have adequate contraception.

Study treatment

Patients received 175 mg/m^2 of paclitaxel over a 3 h infusion on 1 d, followed by nedaplatin 80 mg/m^2 in a 1 h

infusion on 2 d every 3 wk until the documented disease progression, unacceptable toxicity or patient's refusal. These doses were based on a phase I trial of chemotherapy using paclitaxel and nedaplatin in chemotherapy-naive patients with unresectable squamous cell carcinoma (SCC)[22]. Paclitaxel infusions preceded the administration of nedaplatin in the current study, as the interaction of nedaplatin and paclitaxel is highly schedule-dependent[23,24]. As prophylactic agents, dexamethasone (*iv* 20 mg), promethazine (*iv* 25 mg) and cimetidine (*iv* 400 mg) were given 30 min before paclitaxel administration. All patients received adequate antiemetic therapy prior to chemotherapy. Granulocyte colony-stimulating factor was administered at physician's discretion.

Response of treatment and adverse effects

All patients were screened for medical history and underwent a physical examination. Complete blood cell count (CBCC) was performed every week, blood biochemical test and electrocardiogram were performed before every cycle. After every two cycles of treatment, response was evaluated by two independent experts using RECIST criteria. Of the lesions observed prior to treatment, a maximum of five measurable lesions from each metastasized organ up to a total of 10 lesions were selected as target lesions. In the cases of partial response (PR) or complete response (CR), a confirmative computed tomography (CT) scan was performed 4 wk later and this was followed by a CT scan after every two treatment cycles. After discontinuation of treatment, follow-up visits were done every 3 mo to document late toxic effects, disease progression and survival. Toxicity was reported using an NCI-CTC version 3.0 toxicity scale.

Dose modification

The dose of paclitaxel was reduced to 150 mg/m^2 if one of the following conditions occurred: grade 3 neutropenia with infection, grade 4 neutropenia, grade 3 thrombocytopenia or > grade 3 sensory neurotoxicity. If toxicity persisted, a second dose reduction of paclitaxel to 135 mg/m^2 was allowed. In cases of fatigue or asthenia above grade 3, treatment was postponed for 1 wk and restarted when the patient recovered to below grade 2. Patients requiring a delay in therapy for > 2 wk or more than two dose reductions were removed from the study. A new cycle of therapy could begin if the neutrophils count were 1.5 × 10^9/L, the platelets count were 75 × 10^9/L, and all relevant nonhematological toxicities were grade 2. Once a dose had been reduced during a treatment cycle, re-escalation was not permitted during any other subsequent cycles.

Statistical analysis

A Simon's two stage phase Ⅱ design was used. The treatment program was designed to reject response rates of 20% and to provide a significance level of 0.05 with a statistical power of 80% to assess the activity of the regimen at a 40% response rate[25]. The upper limit for a first-stage treatment rejection was 4 responses among 18 evaluable patients; the upper limit of second-stage rejection was 10 responses among 33 evaluable patients. Assuming a drop-out rate of 20%, a total of 39 patients were required. All enrolled patients were included in the intention-to-treat (ITT) analysis of efficacy. Analysis of PFS and overall survival analysis were performed by the Kaplan-Meier method. The PFS was calculated from the initiation of chemotherapy to the date of the disease progression, while overall survival was measured from the initiation of chemotherapy to the date of the last follow-up or death. Statistical data were obtained using an SPSS 11.0 software package (SPSS Inc., Chicago, IL, United States).

RESULTS

Patients' characteristics

Between June 2008 and July 2010, a total of 39 patients from 2 centers (Including Anhui Provincial Hospital affiliated to Anhui Medical University and Anhui Provincial Cancer Hospital) were enrolled. Their baseline characteristics are shown in Table 1.

Efficacy and survival

A total of 39 patients were assessable for response (Table 2). Two patients were not evaluated because of loss to follow-up after two courses, and 1 patient withdrew consent because of toxicities after 1 course. Two patients (5.1%) achieved a CR, 16 patients (41.0%) had PR, 15 patients (38.5%) had SD and 3 patients had PD (7.7%). The median follow-up period was 13.1 mo (range 3.3-28.6 mo). The median PFS for all patients was 7.1 mo (95%CI: 4.6-9.7, Figure 1A). The median OS was 12.4 mo (95%CI: 9.5-15.3, Figure 1B), with a 1-year survival rate of 53.8%.

Adverse events

A total of 141 courses of treatment were given and patients received a median 4 courses (range, 1-6 courses). AE frequencies in this population are listed in Table 3. The most common haematologic AE was leucopenia, which occurred with grade 3/4 in 6 patients (15.4%). Febrile neutropenia was observed in 1 patient (2.6%). Although this case was successfully treated with antibiotics and G-CSF, this patient withdrew his consent after this experience. Grade 3/4 anemia was observed in 3 patients (7.7%) and grade 3 thrombocytopenia in 2 patients (5.1%). Major nonhematologic AEs (in order of decreasing frequency) were nausea (59.0%), fatigue (56.4%), vomiting (46.2%), myalgia (43.6%), alopecia (30.8%), and diarrhea (15.4%). Grade 3/4 nausea vomiting was observed in 4 patients (10.3%) and in 2 patients (5.1%). Hepatic and renal toxicities were mild. No treatment-related death occurred during this study.

DISCUSSION

Esophageal cancer has two main pathological forms: squamous cell carcinoma (ESCC) and adenocarcinoma. Because cardiac adenocarcinoma has been usually classified as gastric cancer, primary esophageal adenocarci-

Table 1 Patients' characteristics (n = 39) n (%)

Characteristics	No. of patients
Age, yr (range)	Median 60 (range, 34-72)
Sex	
Female	1 (2.6)
Male	38 (97.4)
ECOG performance status	
0	4 (10.3)
1	32 (82.0)
2	3 (7.7)
Tumor involved site	
Lymph node	25 (64.1)
Lung	14 (35.9)
Liver	16 (41.0)
Bone	7 (17.9)
Number of involved site	
1	28 (71.8)
2	13 (33.3)
⩾ 3	4 (10.3)
Differentiation	
Poor-differentiated	10 (25.6)
Moderate-well differentiated	23 (59.0)
Unknown	6 (15.4)
Prior treatment (cases)	
Treatment-naive	30 (76.9)
Radiation	4 (10.3)
Operation	5 (12.8)

ECOG: Eastern Cooperative Oncology Group.

noma represents only < 1% of esophageal cancer patients in China[26]. In view of most cases being ESCC, we focused on this type of esophageal cancer in our study. Recurrent or metastatic ESCC remains incurable disease. Systematic combined chemotherapy has been part of combined modality therapy as a palliative treatment for this patient population.

However, there is no standard chemotherapy regimen for advanced esophageal cancer, various kinds of chemotherapy regimens have been tried to prolong survival and improve quality of life. The most commonly used regimen as the first-line chemotherapy is the combination of cisplatin (100 mg/m^2 per day) and 5-FU (1000 mg/m^2 per day continuous infusion for 96-120 h) in metastatic esophageal cancer[27]. The randomized phase Ⅱ study comparing cisplatin/5-FU to cisplatin alone in advanced squamous cell esophageal cancer demonstrated that the combination arm was superior to cisplatin alone arm in terms of RR (35% *vs* 19%, respectively), and OS (33 wk *vs* 28 wk, respectively)[6]. However, high rate of treatment-related deaths (16%) was not acceptable. What's more, continuous infusion of 5-FU requires an indwelling venous access, which provides a source for venous thrombosis and sepsis and makes therapy burdensome to the patient. Until recently, newer agents such as taxanes (paclitaxel and docetaxel), vinorelbine, irinotecan, capecitabine, oxaliplatin and nedaplatin have been investigated as single agent or in combination in neoadjuvant or palliative settings[28].

In the current study, the overall RR was 46.2% (50.0% for 36 valuable patients), the disease control rate was

Table 2 Tumor response (intention-to-treat analysis) n (%)

Response	n = 39
Response rate	
Complete response	2 (5.1)
Partial response	16 (41.0)
Stable disease	15 (38.5)
Progressive disease	3 (7.7)
Not assessable	3 (7.7)

84.6% with a median TTP of 7.1 mo and a median OS of 12.4 mo. This study shows that this regimen has encouraging antitumor activity. Recently, several phase Ⅱ studies were published of paclitaxel and platinum based regimens for advanced or metastatic esophageal cancer[5,8,9,11,12]. Gong *et al*[5] reported that the overall RR was 43.6% and the median progression-free survival (PFS) and OS was 6 and 10 mo, respectively, in a phase Ⅱ study with metastatic esophageal cancer treated with the same combination regimen. Polee *et al*[11] reported that paclitaxel and cisplatin induced a relative longer median PFS of 8 mo, but the median time of OS was only 9 mo. The highest median OS (13 mo) was reported with 7 mo of median TTP by Zhang *et al*[12]. The results of our study can be consistent with those of these published studies.

In consideration of the performance status and chemotherapy tolerance of cancer patients in metastatic setting, treatment related toxicities should be strictly limited. In the present study, the most common grade 3/4 toxicities were leucopenia (15.4%), nausea (10.3%) and anemia (7.7%), thromocytopenia (5.1%), vomiting (5.1%), respectively. Only one patient with febrile neutropenia was discontinued from the study, who was successfully treated with antibiotics and G-CSF. There was no treatment-related death during this study. The toxicities of nedaplatin and paclitaxel regimen were similar with the paclitaxel based regimen reported by Zhang *et al*[12] and Ilson *et al*[10], and more minimal than other studies which applied gemcitabine plus cisplatin, paclitaxel plus carboplatin, nedaplatin plus docetaxel, or irinotecan plus cisplatin/cisplatin-5-FU[9,29-33]. The combination of nedaplatin and paclitaxel was deemed safe in patients with metastatic esophageal carcinoma in spite of the observed toxicity.

So far, some drugs have been applied for advanced esophageal carcinoma, such as capecitabine and oxaliplatin. More recently, a randomized phase Ⅲ trial evaluated capecitabine and oxaliplatin as alternatives to infused 5-FU and cisplatin, respectively, for untreated advanced esophagogastric carcinoma[34]. The more active regimen including epirubicin, oxaliplatin and capecitabine achieved the median PFS of 7 mo and median OS of 11.2 mo, while the relative higher treatment-related toxicities were reported. Of note, all the patients in that study were pathologically confirmed adenocarcinama. So, the standard chemotherapy for advanced or metastatic ESCC still needs more clinical trials.

In conclusion, the results from our phase Ⅱ study demonstrated that the combination of nedaplatin and

Table 3 Adverse events assessment *n* (%)

Adverse event	NCI-CTC grade (n = 39)					Grade 3/4
	1	2	3	4	Any	
Hematologic						
Leucopenia	15 (25.6)	13 (33.3)	4 (10.3)	2 (5.1)	34 (87.2)	15.4%
Anemia	17 (43.6)	5 (12.8)	2 (5.1)	1 (2.6)	25 (64.1)	7.7%
Thrombocytopenia	11 (28.2)	5 (12.8)	2 (5.1)	0 (0.0)	18 (46.2)	5.1%
Nonhematologic						
Gastrointestinal						
Nausea	11 (28.2)	8 (20.5)	4 (10.3)	0 (0.0)	23 (59.0)	10.3%
Vomiting	10 (25.6)	6 (15.4)	2 (5.1)	0 (0.0)	18 (46.2)	5.1%
Diarrhea	6 (15.4)	0 (0.0)	0 (0.0)	0 (0.0)	6 (15.4)	0.0%
Stomatitis	2 (5.1)	1 (2.6)	0 (0.0)	0 (0.0)	3 (7.7)	0.0%
Hepatic						
AST	2 (5.1)	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.1)	0.0%
ALT	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.6)	0.0%
Renal						
Serum creatine	2 (5.1)	0	0 (0.0)	0 (0.0)	2 (5.1)	0.0%
Alopecia	3 (7.7)	9 (23.1)	0 (0.0)	0 (0.0)	12 (30.8)	0.0%
Myalgia	12 (30.8)	5 (12.8)	0 (0.0)	0 (0.0)	18 (43.6)	0.0%
Fatigue	20 (51.3)	2 (5.1)	0 (0.0)	0 (0.0)	22 (56.4)	0.0%
Neutropenia fever	0 (0.0)	0 (0.0)	1 (2.6)	0 (0.0)	1 (2.6)	2.6%

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

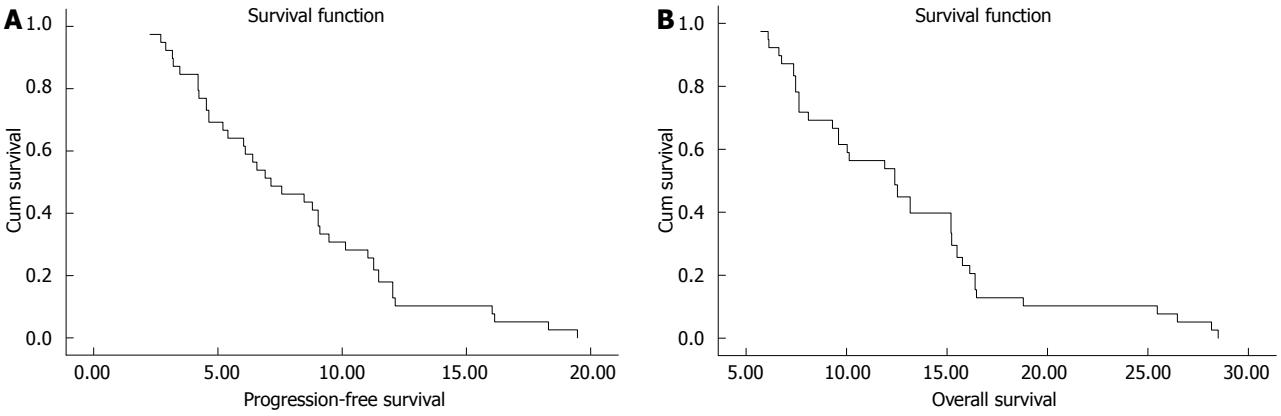

Figure 1 Kaplan-Meier analysis of progression-free survival (A) and overall survival (B) in the study population

paclitaxel is active and well tolerated as a first-line therapy for patients with recurrent or metastatic ESCC. It provides recurrent or metastatic ESCC patients with an effective, safe and convenient chemotherapeutic strategy.

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COMMENTS

Background

Esophageal cancers are among the most aggressive tumors with a poor prognosis. Till now, there has been no standard chemotherapy regimen for advanced esophageal cancer. In this paper, the author conducted a phase Ⅱ study on combination chemotherapy consisting of paclitaxel and nedaplatin in previously untreated patients with metastatic esophageal squamous cell carcinoma (ESCC).

Research frontiers

The most commonly used regimen as the first-line chemotherapy is the combination of cisplatin (100 mg/m^2 per day) and 5-fluorouracil (1000 mg/m^2 per day continuous infusion for 96-120 h) in metastatic esophageal cancer. However, high rate of treatment-related deaths (16%) was not acceptable. So, new regimens were explored to improve the efficacy and safety in metastatic ESCC.

Innovations and breakthroughs

The results demonstrated that the combination of nedaplatin and paclitaxel is active and well tolerated as a first-line therapy for patients with recurrent or metastatic ESCC. It provides recurrent or metastatic ESCC patients with an effective, safe and convenient chemotherapeutic strategy.

Applications

The combination of paclitaxel and nedaplatin is active and well tolerated as a first-line therapy for patients with metastatic ESCC.

Terminology

Paclitaxel, a natural product isolated from the bark of the yew tree Taxus brevifolia, has demonstrated some promising responses against digestive tract cancer. And nedaplatin is a new platinum derivative, selected from a series of platinum analogues based on its pronounced preclinical antitumor activity against various solid tumors with lower nephrotoxicity.

Peer review

This is a good clinical study in which the authors evaluated the efficacy and safety of paclitaxel-nedaplatin combination as a front-line regimen in Chinese patients with metastatic ESCC. The results are interesting and suggest that the combination of the above two drugs is active and well tolerated as a first-line

therapy for patients with metastatic ESCC.

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META-ANALYSIS

Association between vitamin D and hepatitis C virus infection: A meta-analysis

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Abstract

AIM: To evaluate the association between 25-hydroxyvitamin D [25(OH)D] and sustained virological response (SVR) in hepatitis C virus (HCV) infected individuals.

METHODS: Relevant studies were identified by systematically searching MEDLINE databases up to March 2012 and abstracts of the European and American Congress of Hepatology conducted in 2011. Studies must provide information on SVR and the levels of 25(OH)D_3 and/or 25(OH)D_2 [henceforth referred to as 25(OH)D] in sera samples from HCV infected individuals. The inclusion criteria were: clinical studies that included HCV infected patients aged older than 18 years regardless of HCV genotype or ethnic group; provided information on SVR rates; and were reported in the English language as full papers. Due to the heterogeneity of studies in categorizing serum vitamin D levels, a cut-off value of 30 ng/mL of serum 25(OH)D was used. Heterogeneity was assessed using I^2 statistics. The summary odds ratios with their corresponding 95%CI were calculated based on a random-effects model.

RESULTS: Overall, 11 studies (8 observational and 3 interventional) involving 1575 individuals were included and 1117 HCV infected individuals (71%) showed low vitamin D levels. Most of the studies included mono-infected HCV individuals with the mean age ranging from 38 to 56 years. Four studies were conducted in human immunodeficiency virus/HCV infected individuals. Regarding vitamin D measurement, most of the studies employed radioimmunoassays (n = 5) followed by chemiluminescence (n = 4) and just one study employed high performance/pressure liquid chromatography (HPLC). Basal vitamin D levels varied from 17 to 43 ng/mL in the studies selected, and most of the HCV infected individuals had genotype 1 (1068/1575) with mean viral load varying from log 4.5-5.9 UI/mL. With regard to HCV treatment, most of the studies (n = 8) included HCV individuals without previous treatment, where the pooled SVR rate was 46.4%. High rates of SVR were observed in HCV individuals with vitamin D levels above 30 ng/mL (OR = 1.57; 95%CI: 1.12-2.2) and those supplemented with vitamin D (OR = 4.59; 95%CI: 1.67-12.63) regardless of genotype.

CONCLUSION: Our results demonstrated high prevalence of vitamin D deficiency and high SVR in individuals with higher serum vitamin D levels or receiving vitamin D supplementation.

Key words: Vitamin D; Hepatitis C; Therapy; Meta-analysis; Sustained virological response

Core tip: High vitamin D levels (above 30 ng/mL) or

supplementation are associated with sustained virological response in hepatitis C virus infected individuals.

Villar LM, Del Campo JA, Ranchal I, Lampe E, Romero-Gomez M. Association between vitamin D and hepatitis C virus infection: A meta-analysis. *World J Gastroenterol* 2013; 19(35): 5917-5924 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5917.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5917

INTRODUCTION

Viral hepatitis C is a serious public health problem worldwide infecting more than 130 million individuals[1]. Treatment of hepatitis C virus (HCV) infection is usually carried out using pegylated interferon (PEG-IFN) and ribavirin (RBV) for 24 wk for HCV genotypes 2 or 3, or 48 wk for HCV genotype 1 and the main objective of HCV therapy is a sustained virologic response (SVR), defined as an undetectable serum HCV-RNA level at 24 wk after the end of therapy. Rates of SVR range from 60%-70% in chronic hepatitis C (CHC) patients with genotypes 2 and 3, but is less than 50% in patients with genotype 1 using conventional therapy[2].

Recently, studies were conducted to analyze the influence of genetic and metabolic factors in antiviral response[3-5], and a recent review showed that vitamin D levels can influence HCV treatment[6]. Vitamin D itself is considered biologically inactive and is hydroxylated to 25-hydroxyvitamin D [25(OH)D] in the liver. 25(OH)D is the main circulating vitamin D metabolite and is used for classification of the vitamin D status[7,8]. In the kidney, 25(OH)D is converted to 1,25-dihydroxyvitamin D [1,25(OH)D] by 1-alpha-hydroxylase, however, it has been demonstrated that this conversion can occur in many extra-renal tissues including the liver[7,9]. Finally, 25(OH)D or 1,25$(OH)_2$D bind to the ubiquitously expressed vitamin D receptor (VDR), which regulates approximately 3% of the human genome[10]. In this context, vitamin D deficiency has been associated with an increased risk of cancer[7,11], cardiovascular[12,13], autoimmune[14,15] and infectious diseases[6,16].

Due to these facts, there is great research interest in the role of vitamin D status in various infectious diseases. Some studies have shown that high levels of serum vitamin D level are an independent predictor of SVR following anti-viral therapy, and higher SVR is achieved with vitamin D supplementation in CHC individuals[17-22]. However, Lange *et al*[18] found that vitamin D deficiency was associated with a lower SVR rate only in CHC genotype 2/3 patients (treated with PEG-IFN and RBV for 24 wk), but not in CHC genotype 1 patients. Moreover, Jazwinski *et al*[23] found no association between vitamin D levels and SVR in 82 African American genotype 1 CHC-naïve patients, treated with PEG-IFN and RBV.

As vitamin D has an uncertain clinical value in HCV infected individuals and taking into consideration the limitations of previous reviews, we conducted an updated systematic review and meta-analysis to comprehensively assess vitamin D deficiency regarding antiviral therapy and the influence of vitamin D supplementation on SVR.

MATERIALS AND METHODS

Identification of studies

A broad search string was used in MEDLINE in order to identify relevant studies (all languages, all available years, search last completed 31.03.12) using the following search terms: [("vitamin D" [MeSH Terms] or "vitamin D" [All Fields] or "ergocalciferols" [MeSH Terms] or "ergocalciferols" [All Fields]) or ("calcifediol" [MeSH Terms] or "calcifediol" [All Fields] or "calcidiol" [All Fields]) or ("25(OH)D" [All Fields] or "25$(OH)D_2$" [All Fields] or "25$(OH)D_3$" [All Fields]) and [("HCV" [MeSH Terms]) or ("HCV" [All Fields] or "Hepacivirus" [All Fields]) or "Hepacivirus" [MeSH Terms])]. Abstracts from the European and American Association Congress of Hepatology (EASL 2011 and AASLD 2011) were also included in order to give more data on this theme.

Potentially relevant papers were accessed in order to review the abstract and/or full text. Only fully published articles were considered. Duplicate publications were deleted. Two researchers independently performed the literature search and data abstraction with regard to the inclusion and exclusion criteria by reading titles and abstracts. When reading titles and abstracts did not allow identification of eligible studies, articles were read in full. Only original studies conducted in humans were considered for the review. Thus, reviews and letters to the editor were excluded in the analysis, but read in full to identify potential relevant original studies. Disagreements between the two observers were resolved by discussion.

The following data were extracted: year of publication, number of patients, age, vitamin D levels, SVR percentage, method of measurement of vitamin D, HCV genotype, HCV viral load, percentage of naive patients. When such data were not explicitly reported, they were derived from data provided in the articles or requested from the authors through personal contacts, wherever possible.

Eligibility criteria

The study must provide information on SVR against HCV and the levels of 25$(OH)D_3$ and/or 25$(OH)D_2$ [henceforth referred to as 25(OH)D] in sera samples from HCV infected individuals. The inclusion criteria were: clinical studies that included HCV infected patients aged older than 18 years regardless of HCV genotype or ethnic group; provided information on SVR rates; and were reported in the English language as full papers. Studies were excluded if they met the following criteria: they did not provide information on 25(OH)D level, HCV status and/or SVR; basic studies; letters / case reports, or articles not reporting outcomes of interest or

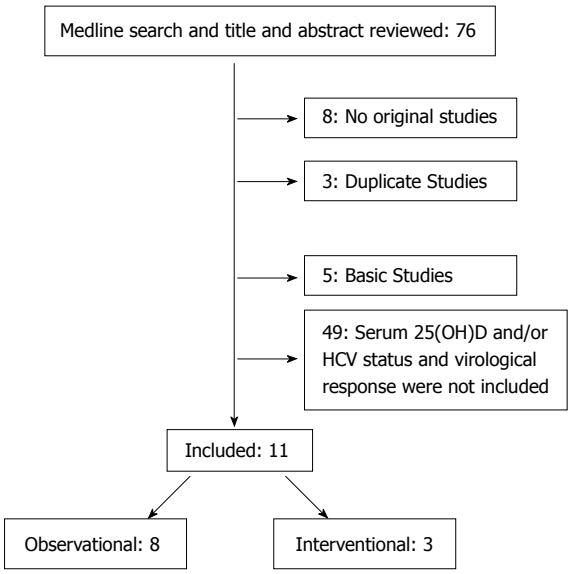

Figure 1 Prisma flowchart for the selection of publications for the systematic review and meta-analysis. HCV: Hepatitis C virus; 25(OH)D: 25-hydroxyvitamin D.

primary data (editorials, reviews).

Statistical analysis

Data were extracted from each paper and compiled for hypovitaminosis D and HCV antiviral response. Statistical analysis was performed using the Meta-Disc software 1.4[24], considering: (1) a summary of data from individual studies; (2) an investigation of the homogeneity of the studies both graphically and statistically; (3) calculation of clustered indices; and (4) exploration of heterogeneity. The meta-analysis was performed using the random-effect model by the Der Simonian and Laird method. Heterogeneity was tested for each planned analysis using the Cochran-Q heterogeneity test and measured using χ^2 and I^2 tests, and statistical significance was considered to be present when $P < 0.05$.

RESULTS

Description of studies included in the meta-analysis

A flow diagram of the search process is shown in Figure 1. The total search yielded 61 articles and 15 abstracts, after accessing the title and abstract, 65 studies were excluded for the following reasons: 49 did not provide data on vitamin D level, HCV status and/or SVR; 5 were basic studies; 8 were reviews, letters or editorials; 3 were duplicate studies.

Eleven studies involving 1575 individuals were included in this study[17,18,20-23,25-29]. The main characteristics of these studies are shown in Tables 1 and 2. Most of the studies were conducted in Europe and only one in North America. Eight studies evaluated vitamin D levels before and after antiviral therapy[17,18,21-23,25,28,29], while three were interventional studies where vitamin D supplementation was conducted[20,26,27]. Most of the studies included mono-infected HCV individuals with the mean age ranging from 38 to 56 years. Four studies were conducted in human immunodeficiency virus/HCV infected individuals[22,25,28,29]. With regard to vitamin D measurement, most of the studies employed radioimmunoassays (n = 5) followed by chemiluminescence (n = 4) and just one study employed HPLC. Basal vitamin D levels varied from 17 to 43 ng/mL in the studies selected, and most of the HCV infected individuals had genotype 1 (1068/1575) with mean viral load ranging from log 4.5-5.9 UI/mL. With regard to HCV treatment, most of the studies (n = 8) included HCV individuals without previous treatment, where the pooled SVR rate was 46.4%.

Vitamin D levels and sustained virological response

Different cut-off values for vitamin D were employed and in order to reduce this heterogeneity, a value of 30 ng/mL was used as the cut-off value, as most of the studies used this value to define vitamin D. Among the observational studies, a total of 1411 individuals were included. Using 30 ng/mL as cut-off value, the χ^2 test of heterogeneity was high (P = 0.3799). There was a significant difference regarding vitamin D levels and SVR, where individuals with values higher than 30 ng/mL had a higher level of SVR. Using the random effects model by the Der Simonian and Laird method, the odds ratio was 1.57 (95%CI: 1.12-2.2) regardless of genotype (Figure 2).

A total of 1117 HCV infected individuals had low vitamin D levels (cut-off value of 30 ng/mL) representing 71% of the population studied, and most of these individuals were in the interventional studies (79.3%) as compared with the observational studies (69.9%). The highest association between vitamin D levels and SVR was observed in the study by Petta *et al*[17] as demonstrated by the OR and CI (OR = 1.96; 95%CI: 1.02-3.79).

Vitamin D supplementation and sustained virological response

With regard to vitamin D supplementation in HCV infected individuals in the interventional studies, the pooled estimation from 3 different studies indicated that SVR rates were higher in treated HCV individuals compared with non-treated HCV individuals. In the meta-analysis of SVR in the interventional studies where the cut-off value was 30 ng/mL, the OR was 4.59 (95%CI: 1.67-12.63) regardless of genotype (Figure 3). The test of heterogeneity (Cochran-Q = 2.86; df = 2; P = 0.2395), inconsistency I^2 = 30%, and t = 0.2454. Of these studies, the OR values were higher in the study where only genotype 1 HCV individuals were included[27] (8.68) compared to the other 2 studies, one study included genotypes 1 and non-1[20] (1.90) and the other study recruited genotype 2 and 3 HCV infected individuals (5.78)[26].

Quality of the studies

Low heterogeneity was observed in the studies included in this meta-analysis according to the Q value for the observational (7.49) and interventional studies (2.86). The possible sources of heterogeneity across the studies were

Table 1 Summary of the general characteristics of the included studies regarding vitamin D and hepatitis C virus (mean ± SD)

Study	Country	Mean age (yr)	Sample Size	Design	25(OH)D measurement	Basal mean vitamin D levels (ng/mL)
Nimer *et al*[26]	Israel	Treated: 48 ± 14 Non treated: 45 ± 10	Treated: 20 HCV infected individuals Non treated: 30 HCV infected individuals	Prospective randomized study	Radioimmunoassay (Diasorin)	Treated: 20 ± 8 Non treated: 19 ± 6
Milazzo *et al*[25]	Italy	45	93 HIV/HCV	Retrospective case-control study (clinical samples)	Radioimmunoassay (IDS)	Cases: 23.1 (15.3-35.3)
Abu-Mouch *et al*[27]	Israel	Treated: 47 ± 11 Non treated: 49 ± 7	Treated: 36 HCV infected individuals Non treated: 36 HCV infected individuals	Prospective randomized study	Radioimmunoassay (Diasorin)	Treated: 19 ± 6 Non treated: 20.5 ± 9
Bitetto *et al*[20]	Italy	Treated: 56 (42-61) Non treated: 52 (23-67)	Treated: 15 HCV infected individuals Non treated: 27 HCV infected individuals	Prospective randomized study	Chemiluminescence immunoassay (Diasorin)	NA
Soumekh *et al*[28]	United States	NA	88 HIV/HCV infected individuals	Prospective study (clinical samples)	Chemiluminescence immunoassay (Diasorin)	NA
Reiberg *et al*[29]	Austria	38	84 HIV/HCV infected individuals	Cohort (clinical sample)	NA	21.9 ± 13.8
Jazwinski *et al*[23]	United States	NA	82 HCV infected individuals	Cohort (clinical sample)	Chemiluminescence immunoassay (Diasorin)	NA
Lange *et al*[18]	Germany	45	468 HCV infected individuals	Cohort (clinical sample)	Radioimmunoassay (Diasorin)	17 (3-80)
Terrier *et al*[22]	France	39.5	189 HIV/HCV infected individuals	Cohort (clinical samples)	Radioimmunoassay (Diasorin)	18.5 ± 9.8
Bitetto *et al*[21]	Italy	47	211 HCV individuals	Cohort (clinical samples)	Chemiluminescence immunoassay (Diasorin)	20.7 (2.1-59.6)
Petta *et al*[17]	Italy	52	Cases: 196 HCV infected individuals	Transversal case-control (clinic and community sample)	HPLC	25.0 ± 9.9

NA: Not available as mean value; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HPLC: High performance/pressure liquid chromatography; 25(OH)D: 25-hydroxyvitamin D.

also explored using meta-regression analysis with the following co-variates as predictor variables: HCV genotype (1 and non-1); previous treatment (yes or no); Origin (Europe or America); method of vitamin D determination (HPLC, chemiluminescence or radiomunoassay). None of these variables interfered with the levels of vitamin D according SVR (data not shown). It is likely that this occurred because most of the studies were from Europe, including HCV genotype I individuals without previous treatment.

Although low heterogeneity was found, it was not possible to ensure high quality of all studies included in this meta-analysis. Some studies did not provide relevant data such as, mean age of the population included[23,28], mean basal vitamin D measurement[20,23,28], or mean HCV viral load[23,26-28].

DISCUSSION

Our review and meta-analysis summarize the results of eleven studies, which included a total of 1575 cases with hepatitis C, where basal 25(OH)D levels and 25(OH)D supplementation were associated with SVR in HCV patients. This updated review confirms and extends earlier results of a systematic review conducted by Cholangitis[6], who reported that vitamin D deficiency is very frequent before liver transplantation and ranges between 51% and 92%, whereas, in the liver transplantation setting, the prevalence of vitamin D deficiency is also high.

Vitamin D is metabolized by the liver and converted to 1,25-dihydroxyvitamin D_3, which is the active form of the vitamin[29,30]. Individuals with chronic liver disease may have poor conversion from vitamin D3 or any of its other biologically active metabolites[31]. Severe liver disease may increase the risk of vitamin D deficiency and/or there might be a relationship between vitamin D deficiency and fibrosis. Putz-Bankuti *et al*[32] and Baur *et al*[33] also showed that low levels of 25(OH)D are associated with fibrosis and suggested that low 25(OH)D levels may predict hepatic decompensation and mortality in patients with chronic liver failure. More recently, Gal-Tanamy *et al*[34] showed that vitamin D_3 increased the expression of the VDR and inhibited viral replication in cell culture.

Due to the observation of vitamin D deficiency in chronic liver disease patients, some studies have been conducted to evaluate vitamin D supplementation in these patients[20,26,27]. In some of these studies, it is reported that higher sunlight exposure or vitamin D supplementation should be recommended in patients with CHC[20,26,27]. In the present meta-analysis, vitamin D supplementation was related to higher SVR rates in HCV infected individuals, where the highest level was observed among genotype 1 HCV infected individuals. Although only a few studies regarding vitamin D supplementation

Table 2 Summary of included studies regarding vitamin D and hepatitis C virus aspects

Study	Sample size	HCV genotype	Mean viral load log, (UI/mL, average)	SVR (*n*)	SVR[1] (above/ below 30 ng/mL)	Previous HCV treatment
Nimer *et al*[26]	50 HCV infected individuals	Ⅱ:28 Ⅲ:22	NA	Treated: 19/20 Non treated: 23/30	19/23	None
Milazzo *et al*[25]	93 HIV/HCV infected individuals	Ⅰ:66 Non Ⅰ:27	5.8 (5.3-6.2)	21	7/14	Naïve: 31 Non responder or relapser to a previous anti-HCV therapy: 20
Abou-Mouch *et al*[27]	72 HCV infected individuals	Ⅰ:72	NA	Treated: 31 Non treated: 15	31/15	None
Bitetto *et al*[20]	42 HCV infected individuals	Ⅰ:32 Non Ⅰ:10	Treated: 5 (3-7) Non treated: 5 (3-7)	Treated: 8/15 Non treated: 5/27	6/7	All
Soumekh *et al*[28]	88 HIV/HCV infected individuals	Ⅰ or Ⅳ:77 Non Ⅰ:11	NA	13	6/7	All
Reiberg *et al*[29]	84 HIV/HCV infected individuals	Ⅰ:47 Non Ⅰ:37	4.5 (1.4-7.6)	39	11/28	None
Jazwinski *et al*[23]	82 HCV infected individuals	Ⅰ:82	NA	74	39/35	None
Lange *et al*[18]	468 HCV infected individuals	Ⅰ:317 Ⅰ:43 Ⅰ:108	5.9 (2.3-7.7)	280	17/152	None
Terrier *et al*[22]	189 HIV/HCV infected individuals	Ⅰ:84 Ⅱ or Ⅲ:73 Ⅳ:31 Other 1	5.9 (0-7)	61	9/52	None
Bitetto *et al*[21]	211 HCV individuals	Ⅰ:95 Ⅱ:63 Ⅱ:38 Ⅳ-Ⅴ:15	5 (2-7)	134	78/56	None
Petta *et al*[17]	196 HCV infected individuals	Ⅰ:196	5 (2-8)	82	26/56	None

[1]According to basal vitamin D cut-off or supplementation. NA: Not available as mean value; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; SVR: Sustained virological response.

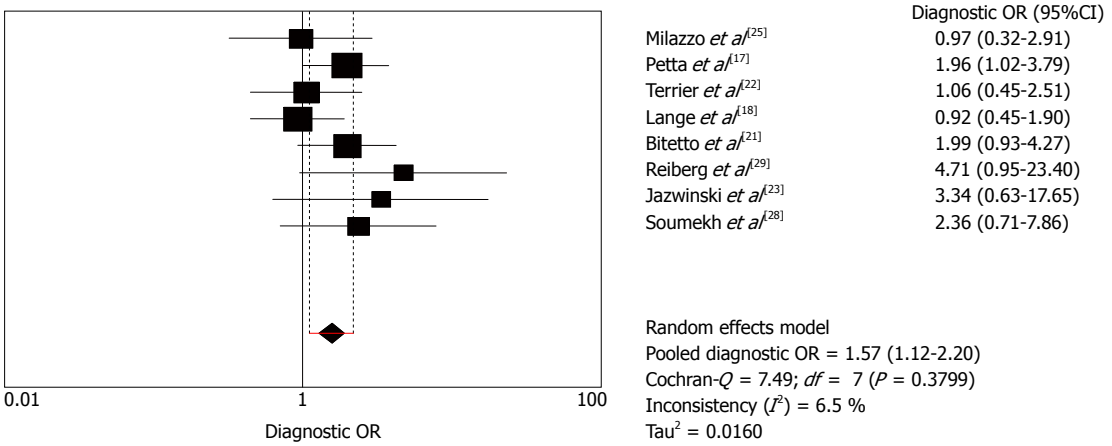

Figure 2 Meta-analysis of 8 observational studies regarding vitamin D levels and sustained virological response against hepatitis C virus infection.

fulfilled the eligibility criteria, different patterns were observed. The first study included only genotypes 2 and 3, the second included only genotype 1 and the third study involved genotypes 1, 2 and 3. Moreover, two of these studies were prospective and one was retrospective. Some limitations of these studies included the small number of patients, lack of vitamin D level assessment during therapy in the treatment and control groups, the design of prospective and randomized studies which were not placebo-controlled in one study[27], and the retrospective design of the study where immunocompromised HCV patients were supplemented with low-dose vitamin D (800 IU/d) after liver transplantation and most of the HCV patients (75%) had low vitamin D levels despite treatment[20].

In this meta-analysis, the levels of vitamin D were also associated with SVR, although different methods of vitamin D determination were used. Lai *et al*[8] demonstrated bias and variability in 25(OH)D measurements between laboratories and between different assays [qui-

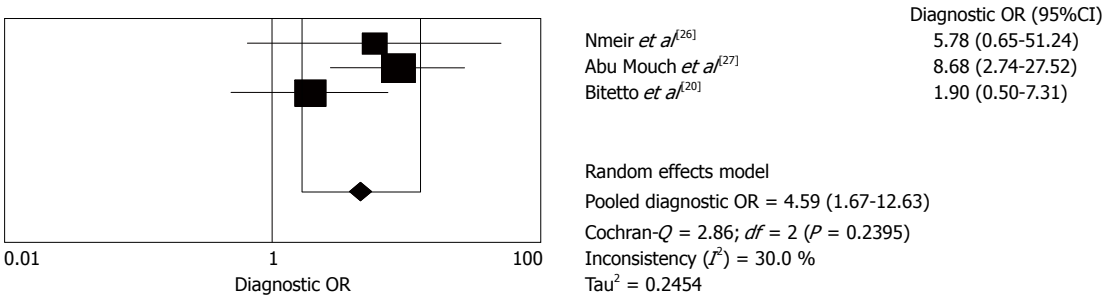

Figure 3 Meta-analysis of 3 interventional studies regarding vitamin D levels and sustained virological response against hepatitis C virus infection.

mioluminescence and liquid chromatography-tandem mass spectrometry (LC-MS/MS)] which can significantly affect clinical decision-making. In this situation, the adoption of common standards to allow assay calibration is urgently required.

Our study is the first meta-analysis of serum 25(OH)D levels and HCV infection in observational and interventional studies. Given the very small numbers of studies available to date, additional studies, ideally from different countries and populations are needed to assess potential differences in the associations between 25(OH)D and SVR for HCV. Large differences can be observed in different populations, depending on exposure to sunlight or vitamin D supplementation, and genetic differences[23]. Moreover, patients of African and Hispanic descent are less likely to respond to standard therapy[23] probably due to polymorphisms of the interleukin (IL)-28B gene, polymorphism of the VDR or vitamin D deficiency[17,35,36]. In this meta-analysis, all the individuals were Caucasian and most lived in Europe, which could explain vitamin D deficiency in this population resulting from possible low exposure to sunlight.

Meta-analysis is an important tool for revealing trends that may not be apparent in a single study. Pooling of independent, but similar studies increases precision and therefore the confidence level of the findings. A particular strength of our study is the application of advanced statistical techniques which allowed a summary of adjusted associations across studies and over the entire range of serum 25(OH)D values, despite the very heterogeneous categorization of 25(OH)D levels in the individual studies. Our study also has important limitations. First, as data on serum 25(OH)D in individuals were not available in each study, median, midpoints and mean of the groups were used for pooling. As a result, estimates of risk may have been less accurate than if data points on each individual had been used. Second, our meta-analysis was limited by the data provided in the individual studies, and although the authors tried to obtain the raw data from the articles, not all were available. Finally, although our review searched the MEDLINE database, recent Congress of Hepatology and Gastroenterology articles, and extensive checks for completeness by cross-referencing were employed, we cannot exclude the possibility that relevant studies may have been missed.

Despite its limitations, our review and meta-analysis support previous suggestions and provide the most comprehensive empirical evidence to date that basal serum 25(OH)D levels and vitamin D supplementation improves SVR in HCV infected individuals. However, available data are still sparse and in-depth analyses of these associations, in the context of additional longitudinal and prospective studies, are highly desirable to enable more precise estimates and a better understanding of the role of vitamin D in HCV infection.

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COMMENTS

Background

Hepatitis C virus (HCV) is a serious public health problem worldwide infecting more than 130 million individuals. Recently, studies have been conducted to analyze the influence of genetic and metabolic factors on antiviral response, and a recent review showed that vitamin D levels can influence HCV treatment.

Research frontiers

Vitamin D itself is considered biologically inactive and is hydroxylated to 25-hydroxyvitamin D [25(OH)D] in the liver. Some studies have suggested that vitamin D deficiency is associated with an increased risk of cancer, cardiovascular, autoimmune and infectious diseases. However, due to the limitations of previous reviews, the authors conducted an updated systematic review and meta-analysis to comprehensively assess vitamin D deficiency with regard to antiviral therapy and the influence of vitamin D supplementation on sustained virological response.

Innovations and breakthroughs

Previous individual studies demonstrated that high levels of vitamin D (above 30 ng/mL) or supplementation are associated to sustained virological response (SVR) in HCV infected individuals. In the present study, a meta-analysis of observational and interventional studies was conducted which proved that high levels of vitamin D (above 30 ng/mL) or supplementation are associated with SVR in HCV infected individuals.

Applications

By showing that basal vitamin D levels or suplementation are important for high rates of SVR in HCV patients, this study may provide a future strategy for therapeutic intervention in the treatment of HCV patients.

Terminology

HCV is an infection caused by a virus transmitted by the parenteral route. Vitamin D itself is considered biologically inactive and is hydroxylated to 25(OH)D in the liver. In the kidney, 25(OH)D is converted to 1,25(OH)$_2$D by 1-alpha-

hydroxylase, however, it has been demonstrated that this conversion can occur in many extra-renal tissues including the liver. Finally, 25(OH)D or 1,25(OH)$_2$D bind to the ubiquitously expressed vitamin D receptor, which regulates approximately 3% of the human genome.

Peer review

The authors examined the influence of vitamin D levels or supplementation among HCV infected individuals. It was observed that high levels of vitamin D or supplementation are strongly associated to SVR among HCV infected individuals. The results are interesting and may represent the role of metabolic factors in HCV infection.

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CASE REPORT

Laparoscopic cholecystectomy for a left-sided gallbladder

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Abstract

Cholecystectomy is a common procedure. Abnormalities in the anatomy of the biliary system are common but an abnormal location of the gallbladder is much rarer. Despite frequent pre-operative imaging, the aberrant location of the gallbladder is commonly discovered at surgery. This article presents a case of a patient with the gallbladder located to the left of the falciform ligament in the absence of situs inversus totalis that presented with right upper quadrant pain. A laparoscopic cholecystectomy was performed and it was noted that the cystic duct originated from the right side. The presence of a left sided gall bladder is often associated with various biliary, portal venous and other anomalies that might lead to intra-operative injuries. The spectrum of unusual positions and anatomical gallbladder abnormalities is reviewed in order to facilitate elective and emergent cholecystectomy as well as other hepatobiliary procedures. With proper identification of the anatomy, minimally invasive approaches are still considered safe.

Key words: Left sided gallbladder; Laparoscopic cholecystectomy; Sinistroposition of the gallbladder; Situs inversus; Bile duct anomaly; Liver anomalies; Portal vein anomaly; Liver transplant

Core tip: In the absence of situs inversus, left sided gallbladders are rare anomalies. They are most commonly encountered during surgery as they usually present with right sided pain and routine preoperative testing fails to identify them. Various biliary, portal venous and other anomalies are associated with left sided gallbladders and their spectrum is reviewed in this article. Recognition of these associated anomalies will help achieve safety in hepatobiliary procedures and prevent injuries.

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INTRODUCTION

Located to the left side of the falciform ligament, left sided gallbladders are rare anomalies and a result of a distinct embryological process. They are seldom identified pre-operatively, and as they are associated with various biliary, portal venous, and other anomalies, the surgeon must be familiar with the potential variations that he might encounter. A left sided gallbladder was encountered during a laparoscopic cholecystectomy at our institution in a patient that presented with right sided abdominal pain. Careful dissection revealed that the cystic duct was crossing from the right side and that critical view was established with the identification of the cystic artery. The spectrum of the possible anomalies associated with left sided gallbladders is wide but does not preclude the successful performance of a minimally invasive cholecystectomy or any other hepatobiliary procedure.

CASE REPORT

The patient is a 64-year-old female with multiple medical problems including type Ⅱ diabetes mellitus, a history of deep venous thrombosis and pulmonary embolism, kidney stones and hypertension who presented to the hospital with a five day history of sharp, right-sided abdominal pain radiating to her epigastric area, chest, bilateral back, right flank and right shoulder. This was the first time patient experienced this kind of pain, and she denied history of prior cholecystitis. She had a history prior extracorporeal shockwave ureteral lithotripsy, but the admitting discomfort was different from that of prior renal colic. Three months prior to the admission, the patient had colonoscopy and esophagogastroduodenoscopy, which were normal.

On physical examination she had stable vital signs and was afebrile. The abdomen was nondistended and nontender. Laboratory data on admission revealed a normal white call count, normal total bilirubin, normal alkaline phosphatase, normal aspartate aminotransferase (AST) and normal alanine aminotransferase (ALT) amylase and lipase were not elevated. An abdominal ultrasound revealed gallbladder sludge with small calculi, no gallbladder wall thickening and no dilatation of the biliary tract. Computed tomography (CT) of the abdomen and pelvis showed gallstones without CT-evidence of cholecystitis, and biliary dilatation up to 9 mm. Secondary to the dilatation of the common bile duct (CBD) on the CT, magnetic resonance cholangiopancreatography (MRCP) was performed and demonstrated cholelithiasis without signs of choledocholithiasis.

During this admission, laparoscopic cholecystectomy was performed. Upon insertion of the camera into the umbilical port, the gallbladder was visualized and was located immediately to the left of the falciform ligament, and below segment Ⅲ of the liver. The gallbladder wall was mildly edematous. The cystic duct and the cystic artery were identified, and it was observed that the artery was to the right of the duct. After the identification of the critical view of safety, the cystic artery and the cystic duct were clipped and divided in a standard fashion. The operation was completed without difficulties and the patient recovered and was discharged home on the second postoperative day. Pathological evaluation identified multiple small, less that 1 mm stones and the thickness of the gallbladder wall measured 3 mm, consistent with chronic cholecystitis and cholelithiasis.

The CT scan of the abdomen did not appear to demonstrate an abnormal location of the gallbladder (Figure 1A) but the finding of the gallbladder to the left of the falciform ligament was present on preoperative MRCP (Figure 1B).

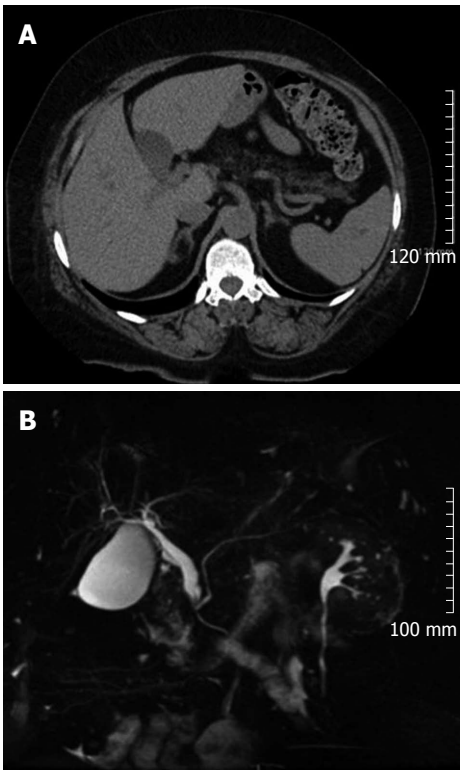

Figure 1 Radiological images of the abdomen. A: Computed tomography scan without contrast demonstrating the gallbladder to the left of the falciform ligament; B: Magnetic resonance cholangiopancreatography showing a dilated common bile duct on coronal view.

DISCUSSION

Left sided gallbladders without situs inversus are rare and have a prevalence of 0.04%-0.3%[1,2]. A distinction should be made between gallbladders that are truly left sided also referred to as sinistroposition and gallbladders located to the left of abnormally located right-sided round ligaments[3]. True left sided gallbladders exist because of two possible embryological etiologies. The first mechanism is due to the attachment and migration of the gallbladder to the left lobe in which case the cystic duct is in a normal anatomic position and crosses in front of the common duct from right to left, as is the case in the case reported herein[4]. The second mechanism is formation of the gallbladder by budding directly from the left side in which case the cystic duct joins the CBD or left hepatic duct from the left side[4,5]. Right-sided round ligaments on the other hand, are associated with a normal position and anatomy of the cystic duct, and with anomalous portal venous branching, which is crucial during the performance a hepatectomy, for example[3].

Despite being truly left-sided, gallbladders with sinistroposition almost always cause right-sided symptoms when they become symptomatic making their preoperative diagnosis difficult[2]. It is believed that the visceral nerve fibers do no transpose with the gallbladder causing right-sided pain[6]. In the present case, the abnormal position of the gallbladder was only discovered at surgery, despite the patient having undergone preoperative ultrasound, CT, MRCP and endoscopic ultrasound, which is consistent with other case reports[2,3,7]. However, an intraoperative finding of a left sided gallbladder should not preclude the decision to proceed laparoscopically with minor modifications in the standard approach and port

Table 1 Literature review of previously reported left-sided gallbladders without situs inversus undergoing surgery

Ref.	Location	Reported number of patients	Clinical presentation	Diagnosis made pre-op?	Surgical treatment (cholecystectomy)	Comments
[1]	Hungary	1 in 2536	Right sided abdominal pain	No	Open	
[2]	The Netherlands	5 in 1764	Right sided abdominal pain	1 of 5	Laparoscopic	Sinistroposition
[3]	Japan	3 in 1621	cholecystitis, 2 incidental during liver surgery	No	Open	Emphasis on right sided round ligament/reported 105 cases in literature until then
[5]	India	Case report	Right sided abdominal pain	No	Laparoscopic	
[6]	India	1 in 1258	Right sided abdominal pain	No	Laparoscopic	Dextrocardia present
[7]	United Kingdom	Case report	Right sided abdominal pain	No	Open	
[11]	Japan	Case report	Incidental/liver cancer	Yes	Open	Used drop infusion cholangiography for diagnosis and CT scan
[12]	Greece	Case report	Epigastric pain	No	Laparoscopic	
[13]	Ohio, United States	Case report	Right sided abdominal pain	No	Laparoscopic	Duplication of CBD
[14]	Serbia	2 patients	1 asymptomatic/1 right sided abdominal pain	No	Open	Associated with liver cysts
[15]	India	Case report	Right sided abdominal pain	No	Open	
[16]	United Kingdom	Case report	Epigastric pain	Yes	Open	Diagnosis by radio-opaque stone on the left side
[17]	Florida, United States	Case report	Right sided abdominal pain	No	Laparoscopic	Intra-op cholangiogram performed
[18]	New York, United States	Case report	Right sided abdominal pain	No	Open	Association with giardia lamblia infection
[19]	Tunis	Case report	Right sided pain	No	Laparoscopic	Normal intra-op cholangiogram
[20]	Japan	Case report	Back pain	No	Laparoscopic	Associated right portal vein anomaly
[21]	St. Louis, United States	Case report	Right sided pain	No	Laparoscopic	CBD injury because of anomalous left sided common hepatic duct
[22]	South Korea	Case report	Right sided pain	No	Laparoscopic	Pre-op percutaneous cholecystostomy with hepatic injury
[23]	Japan	Case report	Right sided pain	Yes	Laparoscopic	Preop diagnosis with DIC CT and lap CBD exploration
[24]	South Korea	3	Omphalocele with herniated liver	Yes	None	Association with omphalocele
[25]	Japan	2	Right sided pain	No	Laparoscopic	
[26]	Japan	Case report	Right sided pain	No	Open	Associated with hypoplasia of the left lobe of the liver
[27]	Japan	Case report	Right sided pain	No	Open	Right sided round ligament
[28]	Japan	Case report	Living donor transplant	No	Open	Association with portal vein anomalous branching
[29]	South Africa	Case report	Right sided pain	Yes	Laparoscopic	Diagnosed on CT pre-op
[30]	China	3	Living donor transplant	Suspected	Open	Biliary, arterial, and portal venous anomalies
[31]	Italy	Case report	Right sided pain	No	Laparoscopic	
[32]	Japan	3	Living donor transplant	Yes	Open	Portal venous anomaly

CBD: Common bile duct; DIC: Drop infusion cholangiography; MRCP: Magnetic resonance cholangiopancreatography; CT: Computed tomography.

placement. Donthi *et al*[8], for example, placed their ports in a mirror image to a typical right-sided standard laparoscopic cholecystectomy, achieving adequate exposure and traction for dissection. Keeping in mind the possible anatomic variations associated with the condition along with careful dissection and the establishment of the critical view of safety, with or without intra-operative cystic duct cholangiography, will minimize complications. Ligation and division of the cystic duct and artery should be close to the gallbladder. The surgeon should make every effort to identify key anatomic landmarks as one would attempt to do during a standard cholecystectomy. Cases of single port cholecystectomy have even been reported in patients with situs inversus without adverse occurrences[9,10]. A more comprehensive literature review of patients with left sided gallbladders without situs inversus undergoing surgery is summarized in Table 1.

In a conclusion, a left-sided gallbladder is an unusual anatomic variant. Patients commonly present with typical biliary colic and cholecystitis symptoms. The abnormal location might not be discovered until the start of the laparoscopic procedure. Proper anatomic identification of key landmarks will permit most or all of these procedures to be performed using minimally invasive techniques.

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CASE REPORT

Totally laparoscopic left hepatectomy using the Torsional Ultrasonic Scalpel

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Abstract

Minimal invasive techniques have allowed for major surgical advances. We report our initial experience of performing total laparoscopic left hepatectomy (segments Ⅱ-Ⅳ) with the Lotus (laparoscopic operation by torsional ultrasound) Ultrasonic Scalpel. The perioperative and postoperative courses of the young female patient were uneventful and she is in a good general condition without complaints 18 mo after surgery. To the best of our knowledge, this is the first total laparoscopic hemihepatectomy to be performed in Greece, as well as the first laparoscopic liver resection using Lotus shears.

Key words: Laparoscopic surgery; Liver resection; Minimally invasive surgery; Hepatectomy; Bloodless surgery; Ultrasonic Scalpel; Ultrasonic dissector; Parenchyma transection; Liver adenoma; Focal nodular hyperplasia

Core tip: This report describes the first total laparoscopic hemihepatectomy performed in Greece, as well as the first laparoscopic liver resection using Lotus shears. The effectiveness of the Lotus Ultrasonic Scalpel highlights the importance of surgical innovation in making minimally invasive procedures available to all surgical specialties.

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INTRODUCTION

The development of minimally invasive hepatic resection techniques in the early 1990s established new surgical standards[1,2] and introduced highly innovative instruments such as ultrasonic dissectors, saline coagulation, and radiofrequency ablation[3-6]. We report our initial experience of performing a laparoscopic left hepatectomy with the ground-breaking Lotus (laparoscopic operation by torsional ultrasound) Ultrasonic Scalpel (S.R.A. Developments, Ashburton, Devon, United Kingdom).

CASE REPORT

A 35-year-old asymptomatic woman with an unremarkable past medical history was referred to our department for surgical management of a liver lesion. The

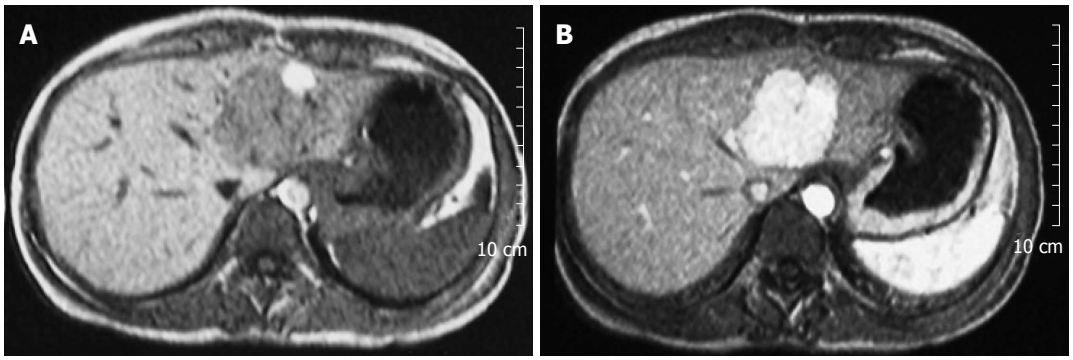

Figure 1 Magnetic resonance imaging showing the liver lesion in segments Ⅲ/Ⅳ. Note the mass effect on the middle and left hepatic veins.

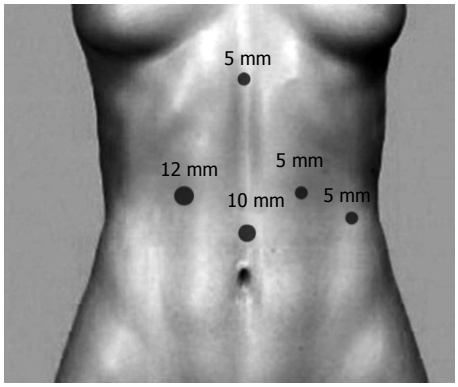

Figure 2 Patient positioning and trocar placement.

tumor had been diagnosed during work-up of elevated γ-glutamyltransferase (GT) (135 U/mL, normal laboratory range 7-36 U/mL) detected at premarital testing. Complete blood count, biochemical profile, liver function tests (except for γGT), and tumor markers were within the normal range. There was no history of oral contraceptive use. Abdominal ultrasound showed a 5-cm isoechoic liver mass in the left hepatic lobe. Gadolinium-enhanced magnetic resonance imaging (MRI) demonstrated a 5.2-cm lesion in segments Ⅲ/Ⅳ, with compression of the middle and left hepatic veins (Figure 1). A laparoscopic left hemihepatectomy was scheduled with a presumed diagnosis of liver adenoma.

Surgical technique

With the patient in the supine position and under general anesthesia[7], five trocar ports were placed as follows: an observation port (10 mm) 4 cm above the umbilicus; a main manipulation port (12 mm) in the midclavicular line below the right costal margin; a 5-mm port below the xiphoid process; and two 5-mm ports (for the assistant surgeon) in the left midclavicular and left anterior axillary lines, respectively (Figure 2). The operating surgeon stood between the patient's legs.

After the falciform and left triangular ligaments were transected, a replaced left hepatic artery branch was identified, clipped, and transected (Figure 3A and B). The left branch of the portal vein was bluntly dissected (Figure 3C) and ligated with an Endopath ETS Articulating Linear Cutter (Ethicon Endo-Surgery, Blue Ash, OH, United States). The liver parenchyma was divided using the Lotus Ultrasonic Scalpel (Figure 3D and E). Non-absorbable clips were used to control the middle hepatic vein, large vessels, intrahepatic bile ducts, and the left hepatic duct. Once this had been achieved, the left hepatic vein was exposed, dissected, and divided with an Endopath ETS Articulating Linear Cutter (Ethicon Endo-Surgery) (Figure 3F). The resected specimen (segments Ⅱ-Ⅳ) was removed *via* a 6-cm supraumbilical incision (Figure 4).

Total operating time was approximately 4 h. Estimated blood loss was < 400 mL. The patient had an uneventful hospital course and was discharged on postoperative day 6. Pathological evaluation of the specimen revealed focal nodular hyperplasia. The patient married 6 mo later and is currently in good health 18 mo after the procedure.

DISCUSSION

Ultrasound-activated scalpels are safe and effective devices[8]. The Lotus Ultrasonic Scalpel introduced the concept of torsional rather than longitudinal ultrasound emissions to achieve transection and hemostasis. Its mechanism of action includes a vibratory grooved blade that generates compression forces directly into the target tissue, and a central blade that cuts as the Teflon jaw is closed. The components of the acoustic systems vibrate harmonically at 36.0 kHz. Laparoscopic torsional ultrasound shears have significant advantages over conventional cutting bipolar forceps when used to divide and coagulate pedicles in gynecological surgery. The Lotus shears are associated with shorter bisection times, less thermal damage, and more effective control of intraparenchymal blood vessels and bile ducts (a major limitation of previous devices).

To the best of our knowledge, this is the first total laparoscopic hemihepatectomy performed in Greece, as well as the first laparoscopic liver resection using Lotus shears. The effectiveness of the Lotus device further emphasizes the importance of surgical innovation in laparoscopic liver surgery.

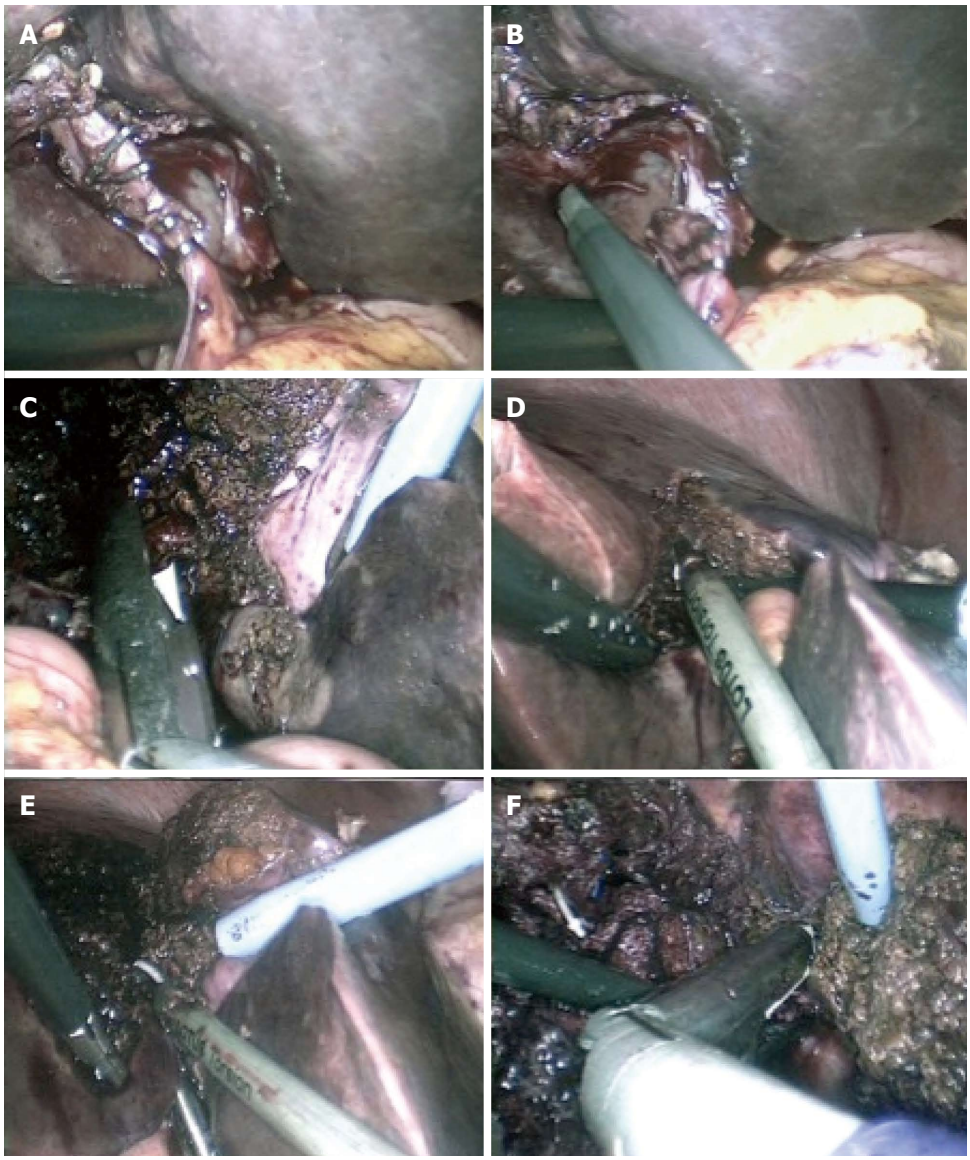

Figure 3 The operation. A and B: Identification, dissection, and clip ligation of the replaced left hepatic artery; C: Dissection of the left portal vein; D and E: Parenchymal transection using the Lotus Ultrasonic Scalpel; F: Dissection of the left hepatic vein.

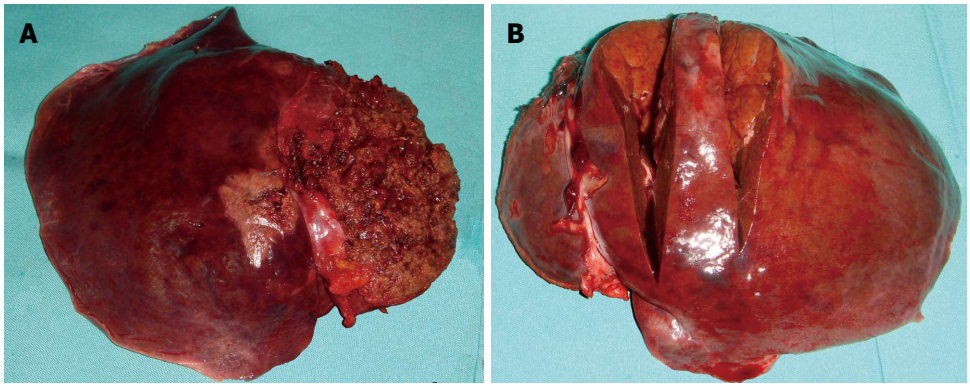

Figure 4 Left hepatectomy specimen (segments Ⅱ-Ⅳ).

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CASE REPORT

Crohn's disease and Takayasu's arteritis: An uncommon association

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Abstract

Takayasu's arteritis (TA) and Crohn's disease (CD) are two rare autoimmune disorders; however some reports describe the presence of both diseases in the same patient. This finding has suggested the possibility that both diseases could share some common etiologic origin. We describe a case of a 13-year-old male affected by CD characterized by fever, diarrhea, weight loss, abdominal pain and elevation of inflammatory markers. Clinical and histological features from colonic specimens were consistent with CD. Treatment with steroids and azathioprine was started, however disease flared every time steroids were tapered. One year later, while still on treatment, he came back to our attention for dyspnea at rest and at night, tiredness and weakness. At physical examination a diastolic heart murmur was found as well as a left carotid artery bruit. A transthoracic echocardiography showed mild aortic valve insufficiency, left ventricular hypertrophy and a dilated ascending aorta with same findings at the aortic arch. A computed tomography scan showed abdominal aorta thickening, dilated thoracic aorta and the presence of a thoracic aortic aneurysm. TA associated with CD was diagnosed and medical treatment with cyclophosphamide, steroids and aminosalicylic acid was started, with good clinical response at 6 mo follow-up. We discuss the presence of possible common causes for the two diseases and the importance of differential diagnosis in those patients characterized for intractable disease.

Key words: Crohn's disease; Takayasu arteritis; Intractable inflammatory bowel disease; children; Treatment

Core tip: It is known that both Takayasu's arteritis (TA) and Crohn's disease (CD) can present together in the same patient although this association is considered extremely rare. We would like to underline the importance of considering an alternative diagnosis in those patients characterized by intractable diseases; in our case, in fact, an intractable CD masked TA and the patient did not achieve clinical remission until he was treated with major immunosupressive therapy; a treatment which can not be considered a standard protocol for CD.

Taddio A, Maschio M, Martelossi S, Barbi E, Ventura A. Crohn's disease and Takayasu's arteritis: An uncommon association. *World J Gastroenterol* 2013; 19(35): 5933-5935 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5933.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5933

INTRODUCTION

Takayasu's arteritis (TA) is a rare, chronic, relapsing large vessel vasculitis affecting the aorta and its major branches, and presenting manifestations include fatigue, weight loss, hypertension, headaches, strokes, and ischemic abdominal pain. Absence of peripheral pulses has given it the name "pulseless disease". Crohn's disease (CD)

is an idiopathic, chronic, relapsing transmural inflammation affecting primarily the gastrointestinal mucosa. The inflammatory process tends to be eccentric and segmental, often with skipped areas (normal regions of bowel between inflamed areas). Both diseases can be considered rare: in the United States, the reported incidence of pediatric CD is 4.56/100000 and the pediatric prevalence is 43/100000[1]; while the incidence of TA is almost 1 case per million. However there are some reports describing the presence of the two conditions in the same patient. It occurs usually during adulthood, while in children this association is extremely rare, but almost 1 in 10 patients with TA may develop CD or CD-like colitis.

Herein we describe the case of a child initially diagnosed as having CD who then presented with TA as well.

CASE REPORT

A 13-year-old-boy presented with a 3 mo history of fever, diarrhea, weight loss and abdominal pain. Laboratory examination revealed elevation of inflammatory markers (erythrocyte sedimentation rate: 110 mm/h; C-reactive protein: 12.5 mg/dL) with microcytic anemia (Hb: 9.2 gr/dL; mean corpuscular volume 68 fl).

Abdominal ultrasound showed an increased terminal ileum wall thickness, while colonoscopy presented linear and aphthous ulcers with some areas of cobblestone mucosa. A biopsy showed the presence of basal plasmacytosis, an increase of lamina propria cellularity (round cells and neutrophils), basal lymphoid aggregates and epithelioid granuloma.

Clinical and histological features were consistent with CD

Treatment with steroids and azathioprine was started, however disease flared every time steroids were tapered. One year later, while still on treatment, he came back to our attention for a clinical picture characterized by dyspnea at rest and at night, with extreme tiredness and weakness. At physical examination a diastolic heart murmur was found as well as a left carotid artery bruit. Transthoracic echocardiography showed mild aortic valve insufficiency, left ventricular hypertrophy and a dilated ascending aorta with same findings at the aortic arch. A computed tomography scan showed abdominal aorta thickening, a dilated thoracic aorta and the presence of a thoracic aortic aneurysm. TA associated with CD was diagnosed and medical treatment with cyclophosphamide, steroids and aminosalicylic acid (ASA) was started with good clinical response at 6 mo follow-up.

DISCUSSION

Although TA is a form of vasculitis that chiefly affects the aorta and its major branches, systemic features such as weight loss, fevers, and fatigue are found in 42%-83% of children at diagnosis of active TA[2]. At the same time musculoskeletal disease, including arthritis, arthralgia, and myalgia, is present in 12%-65% of children as well as skin manifestations, lymphadenopathy posterior reversible encephalopathy syndrome, keratouveitis, bilateral ocular ischemic syndrome and relapsing polychondritis[2].

TA-associated diseases also include pyoderma gangrenosum, ankylosing spondylitis, juvenile rheumatoid arthritis and inflammatory bowel disease[2]. TA in patients with CD was first described in 1970[3], but co-existence of TA and CD has been reported in the following years[4] even if mostly in adulthood, while its presence in childhood is considered extremely rare. The expected prevalence of CD in patients with TA, if present by chance alone, is approximately 0.05%-0.2%. Thus it has been suggested that this unexpected association is more than just a coincidence[5].

Although the pathogenesis of both diseases remains unclear some similarities have been found. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, are common in both and anti-TNF-α monoclonal antibody is an effective therapeutic agent for both TA and CD suggesting the presence of a common inflammatory pathway[5]. In addition, the presence of granulomatous vasculitis was found in 15 out of 25 patients affected by CD[6]; on the other hand the vasculitis of TA is characterized by granulomatous inflammation characterized by transmural inflammation of portions of the arterial wall (including the elastic laminae) and granulomas containing multinucleated histiocytic and foreign body giant cells, histiocytes, lymphocytes (which are predominantly $CD4^+$ T cells), and some plasma cells with fibroblasts[7]. Clinical details of 21 reported cases of TA associated with CD[5,8] showed that CD preceded TA in 13 reported cases as seen in the present patient. In these cases TA developed while being treated with corticosteroids, azathioprine and/or disease modifying drugs, such as 5-ASA, irrespective of the activity of CD and in one case also during infliximab treatment.

We present the interesting case of a patient affected by TA arising some months after CD. Although the exact mechanisms underlying the coexistence of the two diseases is not clear, it seems unlikely that coincidence could account for the simultaneous occurrence of these rare diseases, but the data are insufficient to allow for certainty. As explained above, it seems that a granulomatous inflammation may be considered a final method of development for many different conditions like TA and CD; however chronic granulomatous disease (CGD), Behcet disease or interleukin 17 deficiency may present with the same histological features. On this basis we could speculate that in these patients, inflammatory bowel disease could be considered an intestinal involvement of TA, rather than the coexistence of two different clinical conditions. Unfortunately there are no data on intestinal specimens in patients affected by TA and it is not clear if TA could be considered, in these cases, an extra-intestinal involvement of CD. In addition we would like to underline the importance of considering an alternative diagnosis in those patients affected by CD who do not respond to conventional treatment. In these cases, it is mandatory to rule out the presence of CGD, Behcet disease or TA.

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CASE REPORT

Difficult polypectomy-giant hypopharyngeal polyp: Case report and literature review

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Key words: Esophagus; Giant fibrovascular polyp; Esophageal polyp; Fibroepithelial polyps

Core tip: We report an unusual case of giant hypopharyngeal polyp in a patient with anemia by chronic oozing. Giant esophageal and hypopharyngeal polyps are benign tumors rarely encountered in clinical practice; in fact, there are approximately 250 cases reported in the literature. The interesting fact is the patient regurgitated the polyp during the extraction of the echoendoscope (photo), fortunately without experiencing respiratory distress. It is rare to diagnose these polyps and it is even rarer to perform emergency surgery due to the presence of a large, regurgitated polyp that occupies most of the oral cavity.

Pallabazzer G, Santi S, Biagio S, D'Imporzano S. Difficult polypectomy-giant hypopharyngeal polyp: Case report and literature review. *World J Gastroenterol* 2013; 19(35): 5936-5939 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5936.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5936

Abstract

Giant esophageal and hypopharyngeal polyps are benign tumors rarely encountered in clinical practice. In most cases, they are completely asymptomatic; however, despite the rarity of these tumors, interest in giant esophageal polyps derives from their degree of growth (characterized by slow growth into the esophageal lumen) and their mobility. In fact, if regurgitation occurs, they can ascend into the oral cavity and be aspirated into the airways, with potentially lethal consequences. The removal of these giant polyps is recommended. An adequate preoperative evaluation to identify the correct origin of the stalk is mandatory for a successful endoscopic or surgical treatment. A 60-year-old man was admitted to our hospital for anemia. The patient underwent gastroscopy, contrast computed tomography and endoscopic ultrasound. At the conclusion of the procedure, during the extraction of the echoendoscope, the patient began retching and regurgitated the polyp, without experiencing respiratory distress. The patient underwent a left cervicotomy and polyp dissection *via* a pharyngotomy.

INTRODUCTION

Giant esophageal and hypopharyngeal polyps are benign tumors rarely encountered in clinical practice; they represent approximately 0.03% of all esophageal/hypopharyngeal neoplasms[1]. The majority originate from the upper third of the esophagus, rarely from the hypopharynx, and extremely rarely from the oropharynx. They may vary greatly in size; however, they can extend along the entire length of the esophagus until reaching the gastric cavity. In most cases, they are completely asymptomatic; however, they rarely can cause anemia by chronic oozing. In addition, they can cause asphyxiation by regurgitation of the polyp into the oral cavity. The world literature contains about 110 cases of giant oropharyngeal or esopha-

geal polyps (> 5 cm); they are most often successfully treated by peroral, cervicotomic, or thoracotomy surgery; they also can be managed by endoscopy[2].

CASE REPORT

A 60-year-old man, with a negative medical history, was admitted to another hospital eight months ago for anemia and underwent colonoscopy (findings: negative), gastroscopy (findings: esophageal neoformation of the lower third of the esophagus), and computed tomography (CT) (findings: submucosal esophageal neoformation capturing contrast medium, extending from the cervical to the cardiac esophagus). After transfusion therapy, the patient refused any surgical treatment. He enjoyed good health until another episode of anemia occurred (hemoglobin 8.6 g/dL) and he presented at our facility. The patient underwent a gastroscopy (findings: esophageal stenosis for submucosal neoformation arising from the cervical esophagus, occupying approximately 50% of the esophageal lumen, with mucosal integrity, and protrusion of a neoformation with erosion of its apex into the gastric cavity). He also underwent a contrast CT (findings: bulky neoformation jutting into the esophageal lumen extending from the cervical to the distal esophagus with a maximum diameter of 35 mm × 46 mm, associated with increased esophageal diameter) (Figure 1). The patient then underwent an endoscopic ultrasound (EUS) (findings: thickening of the esophageal wall due to a solid, hypoechoic, neoformation, with hyperechoic areas and a vascular network, originating from the submucosa of the cervical esophagus, which affected the esophagus from cardiac portion to the upper esophageal sphincter) (Figure 2). At the conclusion of the procedure, during the extraction of the echoendoscope, the patient began retching and regurgitated the polyp, without experiencing respiratory distress (Figure 3). The patient was taken to the operating room and, under general anesthesia, a gastroscopy was repeated. It revealed that the origin of the polyp was not at the upper esophageal sphincter (UES), as previously diagnosed by CT, EUS, and the first gastroscopy; rather, it originated in the hypopharynx below the left arytenoid cartilage, with a stalk of about 3 cm in length. The patient underwent a left cervicotomy and polyp dissection *via* a pharyngotomy. The removal of the polyp was performed with a stapler (Endogia 45, Ethicon®). At the conclusion of the procedure, a dual lumen nasogastric tube was placed. On the seventh postoperative day the patient underwent a radiological evaluation with water-soluble contrast medium, which imaged good transit in the absence of extraluminal spillage. The patient began a semi-liquid diet and was discharged on the 10th postoperative day. A gastroscopic evaluation 45 d postoperatively was normal.

DISCUSSION

Benign esophageal/hypopharyngeal tumors are rare and represent less than 1% of esophageal/hypopharyngeal neoplasms, *e.g.*, Moersch and Harrington[3] discovered 44 (0.59%) benign esophageal tumors in 7459 consecutive autopsies at the Mayo Clinic. Most of these tumors are intramural, represented by leiomyomas, neurofibromas, and hemangiomas; the intraluminal lesions are represented by fibrolipomas, fibromixomas, hamartomas, fibromas, and lipomas[4]. These tumors are globally ranked by the World Health Organization as fibrovascular polyps. Histologically, giant esophageal polyps are covered by

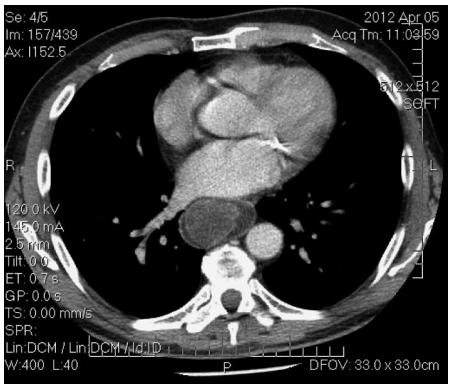

Figure 1 Computed tomography (mediastinal window setting) shows bulky neoformation jutting into the esophageal lumen.

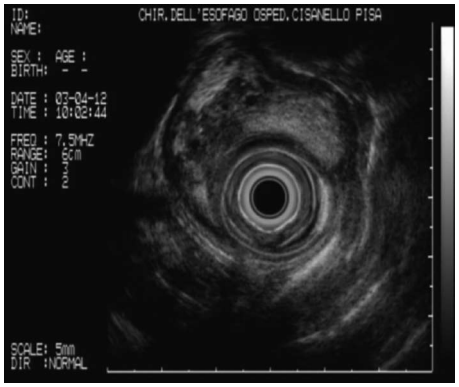

Figure 2 Endoscopic ultrasound shows a solid, hypoechoic, neoformation, with hyperechoic areas and a vascular network.

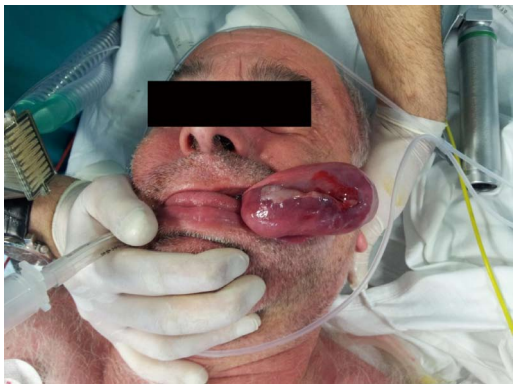

Figure 3 Giant, regurgitated hypopharyngeal polyp.

squamous epithelium with a fibrovascular axis, consisting of adipose and connective tissue to varying degrees, and a well-developed vascular network. Despite the rarity of these tumors, interest in giant esophageal polyps derives from their degree of growth (characterized by slow growth into the esophageal lumen) and their mobility. In fact, if regurgitation occurs, they can ascend into the oral cavity and be aspirated into the airways, with potentially lethal consequences. They are more frequent in males (male:female ratio = 3:1)[4], and are usually diagnosed in the sixth or seventh decade of life; however, fibrovascular polyps have been reported in childhood[5]. About 85% are located in the upper third of the esophagus, close to the UES, and originate as small mucosal tumors, extending into the esophageal lumen due to the constant downward thrust by food ingestion and peristalsis. In the present case, the polyp was attached in an unusual site, at the level of the left anterolateral hypopharyngeal wall immediately below the ipsilateral arytenoid cartilage, with dimensions of 18 cm × 5.4 cm × 4 cm. Fibrovascular polyps usually occur as solitary lesions; however, some authors have reported synchronous polyps[6]. Due to progressive tumor growth, most patients report progressive dysphagia, initially with solid food and then with liquids. The second most frequent symptom is the regurgitation of the polyp into the hypopharynx or oral cavity with the risk of aspiration into the airways, resulting in asphyxia[7-10]. In a small percentage of patients, aspiration of the polyp may be the presenting symptom. Other symptoms include pharyngeal globus, weight loss, odynophagia, pharyngodynia, vomiting, abdominal pain, gastroesophageal reflux, hiccups, melena, and anemia[4,11]. The latter two symptoms are a result of ulceration of the apical part of the polyps due to gastric acidity or reflux of gastric contents into the esophagus. Malignant degeneration of these polyps is rare.

Fibrovascular polyps can be detected by either a barium esophagogram or gastroscopy. The former may reveal a dilated esophagus, with a gross intraluminal defect usually arising in proximity to the UES. However the examination may be entirely negative especially if the polyp remains in contact with the esophageal wall. The diagnosis, however, by gastroscopy, sometimes may be difficult or impossible because the fibrovascular polyp can completely or partially occupy the esophageal lumen, move against the esophageal wall and thus present a similar appearance to the mucosa. Diagnostic suspicion can be confirmed with a rear view, because if the terminal part of the polyp protrudes into the gastric cavity, it may image as a "clapper of a bell"; thus illustrating the circumferential space between the two walls. EUS may be useful for diagnosis because it clearly highlights the fibrovascular axis of the polyp, the echogenic aspect of adipose tissue, and the presence of anechoic areas due to its vascular network. Finally, this imaging procedure can help in a diagnosis *via* a needle aspiration. CT and magnetic resonance (MR) can be useful in confirming diagnostic suspicion and in deciding the proper surgical approach. In particular, MR with axial coronal and sagittal scans, allows a precise identification of the stalk, an essential requirement for proper treatment. Despite a careful diagnostic process, fibrovascular polyps can be confused with intramural lesions; thus, identification of the stalk can be difficult and incorrect. In a review by Caceres *et al*[2], 22% of barium esophagograms and 33% of gastroscopies yielded false negative results. Due to the potentially disastrous complications, removal of benign esophageal and hypopharyngeal polyps is strongly recommended. This can be achieved using a transoral, transcervical, transthoracic, or endoscopic approach, depending on the location and size of the polyp.

Two field approaches (abdominal and cervical) have also been described in patients with unusually large polyps[12]. It is clear that the correct diagnosis is essential to avoid unnecessary intervention and especially to choose the type of the intervention: surgical or endoscopic. For example, Oh *et al*[13] reported some difficulty in removing a cervical esophageal fibrovascular polyp through a right thoracotomy. Most polyps are surgically removed, especially those with a large, vascularized stalk; however, it has been performed endoscopically by dissection of the peduncle by ligature, electrocoagulation, or laser[14,15]. Generally, the removal of the polyp is curative and recurrence after resection is rare; however, some authors have reported a recurrence[16,17].

In conclusion, giant esophageal polyps are extremely rare, benign tumors, whose removal is recommended because of the possibility of fatal consequences, bleeding and malignant transformation (rare). An adequate preoperative evaluation to identify the correct origin of the stalk is mandatory for a successful endoscopic or surgical treatment. In addition to the removal of the giant polyp, all mucosal redundancy must be evaluated and possibly removed to avoid recurrences, which are rare but possible.

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CASE REPORT

Obscure bleeding colonic duplication responds to proton pump inhibitor therapy

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Author contributions: Jacques J and Sautereau D reported this case; Jacques J and Loustaud-Ratti V wrote the paper; Jacques J, Legros R, Sarabi M, Carrier P and Valgueblasse V were attending doctors for the patient; Bouvier S and Fredon F performed the surgical operation; Projetti F performed pathological examinations.

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Abstract

We report the case of a 17-year-old male admitted to our academic hospital with massive rectal bleeding. Since childhood he had reported recurrent gastrointestinal bleeding and had two exploratory laparotomies 5 and 2 years previously. An emergency abdominal computed tomography scan, gastroscopy and colonoscopy, performed after hemodynamic stabilization, were considered normal. High-dose intravenous proton pump inhibitor (PPI) therapy was initiated and bleeding stopped spontaneously. Two other massive rectal bleeds occurred 8 h after each cessation of PPI which led to a hemostatic laparotomy after negative gastroscopy and small bowel capsule endoscopy. This showed long tubular duplication of the right colon, with fresh blood in the duplicated colon. Obscure lower gastrointestinal bleeding is a difficult medical situation and potentially life-threatening. The presence of ulcerated ectopic gastric mucosa in the colonic duplication explains the partial efficacy of PPI therapy. Obscure gastrointestinal bleeding responding to empiric anti-acid therapy should probably evoke the diagnosis of bleeding ectopic gastric mucosa such as Meckel's diverticulum or gastrointestinal duplication, and gastroenterologists should be aware of this potential medical situation.

Key words: Colonic duplication; Gastro-intestinal duplication; Gastrointestinal bleeding; Hemostatic colorectal surgery; Proton pump inhibitor therapy

Core tip: Obscure lower gastrointestinal bleeding is a difficult medical situation and potentially life threatening. The collaboration between endoscopists and radiologists usually allow location of the source of bleeding, but some rare situations, such as gastrointestinal malformations, need surgical intervention to diagnose and concomitantly treat an obscure bleeding source. In terms of medical therapy, only proton pump inhibitor therapy has efficacy in peptic ulcer disease. Obscure gastrointestinal bleeding responding to empiric antacid therapy should suggest the diagnosis of bleeding ectopic gastric mucosa such as Meckel's diverticulum or gastrointestinal duplication, and gastroenterologists should be aware of this potential medical situation.

Jacques J, Projetti F, Legros R, Valgueblasse V, Sarabi M, Carrier P, Fredon F, Bouvier S, Loustaud-Ratti V, Sautereau D. Obscure bleeding colonic duplication responds to proton pump inhibitor therapy. *World J Gastroenterol* 2013; 19(35): 5940-5942 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5940.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5940

INTRODUCTION

Obscure lower gastrointestinal bleeding is a difficult medical situation which is potentially life-threatening. No

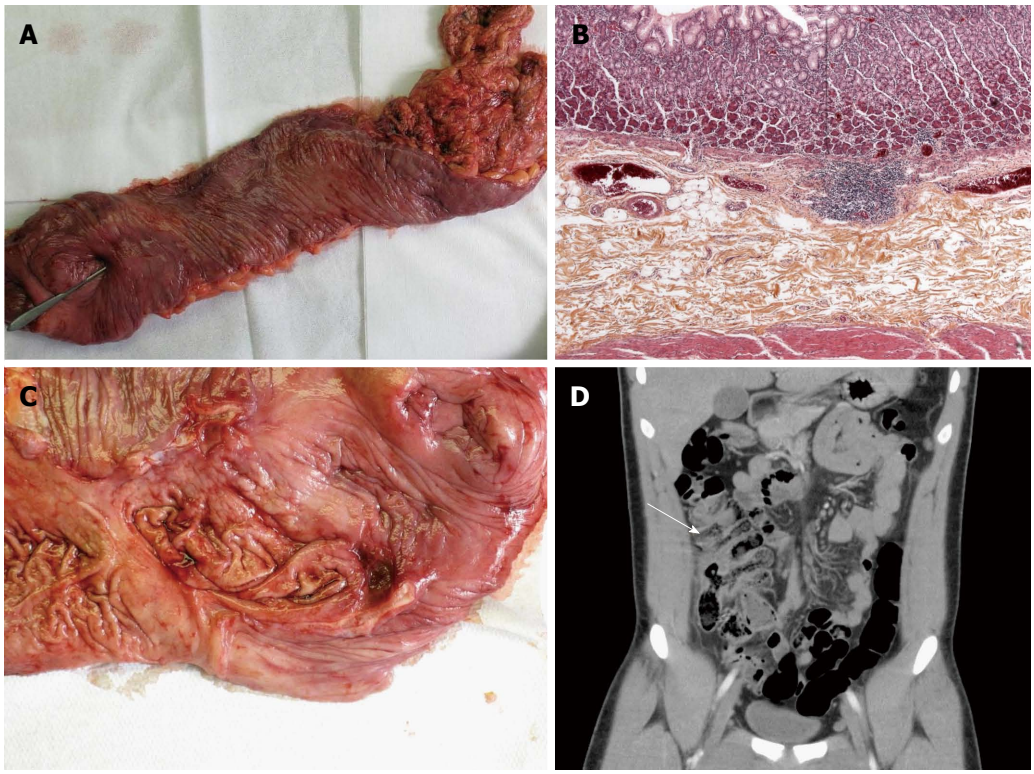

Figure 1 Colonic duplication. A: Macroscopic analysis of the resected colonic duplication: colonic duplication with a pen showing the anastomosis with the right colon; B: Pathological analysis of the duplication: pathological analysis showing intestinal duplication with a smooth muscle coat and an ectopic gastric mucosal lining; C: Bleeding ulcer in the colonic duplication: macroscopic view of a large bleeding ulcer near the anastomosis between the duplicated and the right colon; D: Computed tomography scan coronal view of the colonic duplication: retrospective coronal reconstruction of the emergency computed tomography scan showing the duplicated right colon (arrow) which was not visible in the axial view.

medical therapy is currently available to manage these patients until endoscopic, radiologic or surgical therapy is performed after the identification of the bleeding lesion. Proton pump inhibitor (PPI) therapy is not recommended for bleeding lesions downstream of the angle of Treitz. We describe here the partial efficacy of PPI therapy in massive obscure gastrointestinal bleeding due to a bleeding ulcer in the ectopic gastric mucosa of an undiagnosed colonic duplication.

CASE REPORT

A 17-year-old male was admitted to the emergency room of our academic hospital with massive rectal bleeding. He had reported recurrent gastrointestinal bleeding since childhood, which led to multiple hospitalizations. At 5 and 2 years before this admission he underwent exploratory laparotomies because of suspicion of a Meckel diverticulum. On admission, blood pressure was lowered to 80/55 mmHg, with a reflex tachycardia of 120 beats/min. The hemoglobin level was 5.7 g/dL. Initial resuscitation required significant volume replacement, and the transfusion of 3 blood units and 3 fresh bags of frozen plasma. High-dose intravenous PPI therapy at the dosage used in peptic ulcer bleeding (8 mg/h) was initiated.

An emergency abdominal computed tomography (CT) scan, performed after hemodynamic stabilization, was considered normal. A gastroscopy and an ileocolonoscopy, with a water-jet device after preparation by an enema, were normal. Bleeding stopped spontaneously, and after a 24-h clinical and biological stabilization, we decided to stop the PPI therapy because of the absence of gastroduodenal ulcer disease.

Unfortunately, a further massive rectal bleed consisting of red fresh blood with melena, and hemodynamic instability occurred 8 h later. High dose intravenous PPI therapy was reintroduced and gastroscopy conducted by a French national expert in endoscopy was once again considered normal. Small bowel capsule endoscopy was then performed and his initial interpretation eliminated a bleeding lesion upstream of the angle of Treitz. PPI therapy was once again stopped and 8 h later a third massive rectal bleed occurred which led to selective mesenteric arteriography. This showed suspicious hypervascularization of the right colon. Hemostatic laparotomy was performed because of the patient's unstable condition and lack of a diagnosis. This showed long tubular duplication of the right colon, which was connected to the cecum at one end (Figure 1A), with fresh blood in the duplicated colon. Although anastomosis was not visible during the endoscopy, the capsule was seen inside the duplicated colon. Hemostatic right hemicolectomy with ileocolonic anastomosis was performed. Pathological analysis (Figure 1B) showed intestinal duplication, with intimate attachment to the mesentery, a smooth muscle coat and an ectopic gastric mucosal lining. A large ulcer with stigma of recent hem-

orrhage (Figure 1C) was present near the anastomosis. Retrospective analysis of the CT scan by a senior gastrointestinal radiologist using coronal reconstruction agreed with our diagnosis as difficult and rare, but possible (Figure 1D, arrow). Two years after the surgery, the patient had no further gastrointestinal bleeding.

DISCUSSION

Duplications of the gastrointestinal tract are rare congenital anomalies that can occur anywhere along its length[1,2], and are characterized by attachment to at least one part of the tract, a layer of smooth muscle and epithelial lining resembling some part of the tract[3]. Colonic duplication is a rare abnormality, accounting for 4%-18% of all gastrointestinal duplications, and is usually located in the cecum. Tubular types are found in only 18% of cases and are most often encountered in the small and large bowel. Gastric mucosa is frequently observed in the wall of the duplications, irrespective of their site of origin in the alimentary tract[3]. Clinically, they can become a problem at any age, but the majority are discovered during the neonatal period or infancy[4,5]. They can manifest as abdominal masses[6], bowel obstruction and gastrointestinal hemorrhage[7]. A diagnosis of intestinal duplication can be difficult and is usually performed intraoperatively.

This case highlights the diagnostic and therapeutic evaluation during massive gastrointestinal bleeding of unknown origin. First, despite all diagnostic tools available nowadays in a tertiary center, obscure gastrointestinal bleeding can sometimes be a diagnostic and therapeutic challenge. The growing efficacy of endoscopic devices (gastroscopy, colonoscopy with washing-pump, small bowel capsule endoscopy and enteroscopy) and radiologic procedures (high resolution CT scan, arteriography) tend to make the surgical procedure therapeutic only. This case highlights the unusual but still existing role of surgery in the diagnosis and treatment of massive gastrointestinal bleeds of unknown etiology[8] in challenging cases. Whereas such a role is common in cases of obscure bleeding during childhood, the usefulness of surgical exploration must not be ignored in adults. Gastrointestinal congenital malformations such as colonic duplication are a rare cause of obscure gastrointestinal bleeding. Coronal and sagittal reconstruction of CT scans can be very useful and must always be made in cases where there is an absence of diagnosis.

In terms of medical therapy, PPI therapy represents the treatment of choice in non-portal hypertensive gastrointestinal bleeding of the upper gastrointestinal tract[9]. No drugs are approved nowadays for the management of lower gastrointestinal hemorrhage[8]. In this case, the discrepancy in the efficacy of PPI therapy and no sign of a gastroduodenal ulcer is explained by the pathology result. High dose PPI therapy probably acted on the bleeding ulcer existing in the ectopic gastric mucosa in the duplicated right colon. Rare cases have already been reported on the hemostatic efficacy of anti-acid therapy (H2-receptor antagonist or PPI) in bleeding Meckel's diverticula whose histological analysis reveals ectopic gastric mucosa in more than half of the cases[10]. Obscure gastrointestinal bleeding responding to empiric anti-acid therapy should probably suggest the diagnosis of bleeding ectopic gastric mucosa such as Meckel's diverticulum or gastrointestinal duplication.

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Capsule-odometer: A concept to improve accurate lesion localisation

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Abstract

In order to improve lesion localisation in small-bowel capsule endoscopy, a modified capsule design has been proposed incorporating localisation and - in theory - stabilization capabilities. The proposed design consists of a capsule fitted with protruding wheels attached to a spring-mechanism. This would act as a miniature odometer, leading to more accurate lesion localization information in relation to the onset of the investigation (spring expansion *e.g.*, pyloric opening). Furthermore, this capsule could allow stabilization of the recorded video as any erratic, non-forward movement through the gut is minimised. Three-dimensional (3-D) printing technology was used to build a capsule prototype. Thereafter, miniature wheels were also 3-D printed and mounted on a spring which was attached to conventional capsule endoscopes for the purpose of this proof-of-concept experiment. *In vitro* and *ex vivo* experiments with porcine small-bowel are presented herein. Further experiments have been scheduled.

Key words: Capsule endoscopy; Odometer; Localisation; Hardware; Software

Core tip: In order to improve localisation, capsule endoscopy developers proposed the use of an applied external magnetic field. However, this affords only a rough estimate of capsule - hence lesion - localization. Therefore, a modified capsule design was proposed in 2010. It consists of a capsule fitted with protruding wheels attached to a spring-mechanism. This allows the wheels to retract or expand to fit the lumen, whilst the capsule passes through the intestine, acting as a miniature odometer. Using three-dimensional printing technology, we built a conceptual capsule prototype and miniature wheels; with the latter, we perform *in vitro* and *ex vivo* proof-of-concept experiments.

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TO THE EDITOR

Since its commercialization in the early 2000s, wireless capsule endoscopy (CE) has become a mainstream investigation for the small-bowel[1]. However, as with any technological advancement, there are performance limitations. In CE, the two main issues are low video resolution and reduced image capture rate[2]. Additionally, current systems offer somewhat crude localisation software - of

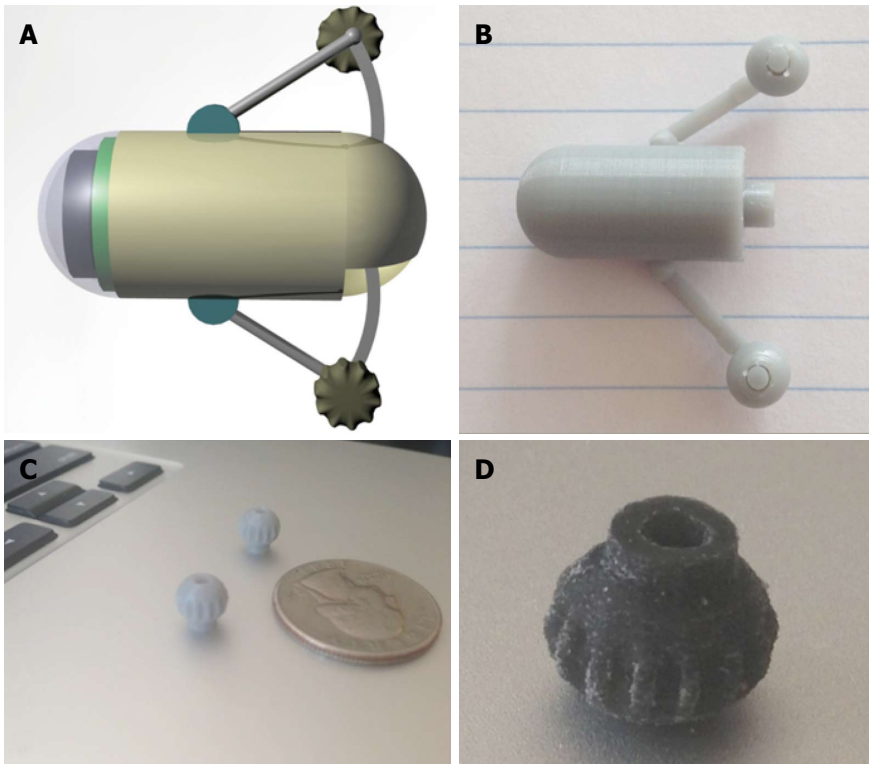

Figure 1 Capsule odometer,conceptual design and its three-dimensional printing realisation. A: Conceptual design[10] of a capsule endoscope (herein called ODOcaps) with protruding wheels attached to a spring-mechanism; B: Three-dimensional printing technology used to build a capsule prototype; C: Wheels were initially produced with UV Curable Acrylic Plastic; D: Wheels later made of a synthetic resilient, textile-like material (TPU 92A-1).

questionable help - in mapping the imaged small-bowel. To address these issues, "smarter" CE designs have been proposed using enhanced digital circuits[2] with more efficient antennae[2-7], and on-board video-compression[8] to conserve battery energy and increase video resolution and image capture frame rate.

In order to improve capsule localisation, capsule endoscope developers have proposed the use of an applied external magnetic field powerful enough to manoeuvre a capsule[9]. In addition, by using geometric algorithms and applying magnetic forces, measurement of the location of the capsule as a vector in the coordinate's space is achievable[9]. However, this method affords only a rough estimate of capsule position due to: (1) constant changes in the anatomy of the human intestine; and (2) all measurements are taken relative to the externally applied magnetic field.

In 2010[10], we proposed a modified capsule design incorporating localisation and stabilization capabilities. The proposed design consists of a capsule fitted with protruding wheels attached to a spring-mechanism (Figure 1A). These springs allow the wheels to retract or expand to fit the lumen whilst the capsule passes through the intestine. This would then lead to more accurate location information, hence accurate lesion localisation, in relation to the onset (pyloric opening) of the investigation. Furthermore, this capsule could theoretically allow stabilization of the recorded video as any erratic, non-forward movement through the gut is minimised.

Therefore, we aim to test the feasibility of the proposed design *in vitro* and *ex vivo*. Three-dimensional (3-D) printing technology[11,12] was used to build a conceptual capsule prototype (herein called ODOcaps) (Figure 1B). Furthermore, miniature wheels were initially produced with UV Curable Acrylic Plastic (Figure 1C). They were later re-designed and made of a synthetic resilient, textile-like material, chosen for its known strength, flexibility and durability (TPU 92A-1) (Figure 1D). This material is produced *via* laser sintering, a process similar to stereo-lithography that uses temperature-sensitive powder (instead of UV-sensitive liquid), causing the powder to become a coherent mass without melting[13]. For the tread area of the wheels, two designs were considered: smooth or tractor-tread and tested on various types of surfaces. The tractor-tread design of the wheels was selected because it could - theoretically - achieve better traction and full rotation of the wheels when in contact with the intestinal mucosa.

Thereafter, the wheels were inserted in 3-D printed, L-shaped miniature tubes (UV Curable Acrylic Plastic; Figure 2A) allowing almost frictionless rotation. The tubes along with the wheels where attached to a spring (stainless steel torsion spring[14] with 90° deflection and 0.093 inch pounds torque from Associated Spring Raymond) to allow extension/retraction of the wheels, Figure 2B. Thereafter, spring with wheels was clipped onto a 3-D printed ring (11.5 mm in diameter made of UV Curable Acrylic Plastic; Figure 2B and C). The ring has two (1 mm) holes, diametrically opposite to each other. The ring was designed to tightly fit on conventional CE

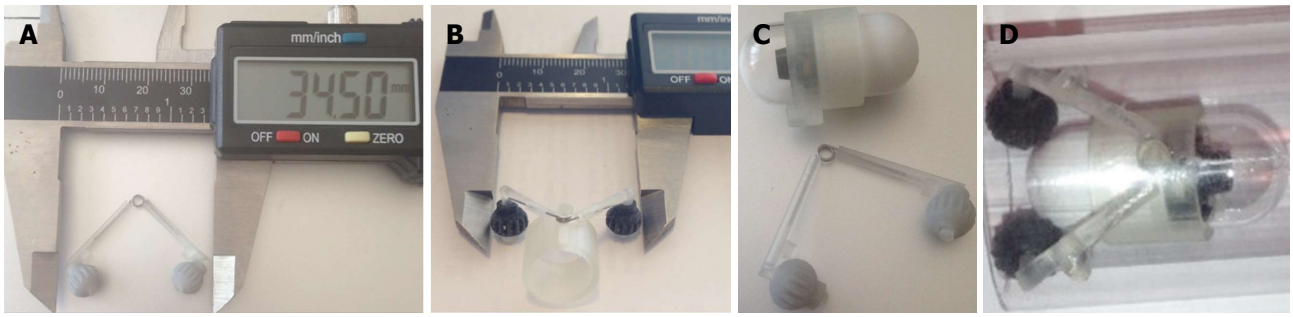

Figure 2 ***In vitro*** **experiment.** A: Wheels inserted in three-dimensional printed, L-shaped miniature tubes. Tubes along with the wheels attached to a spring; B: stainless steel torsion spring with 90° deflection and 0.093 inch pounds torque from Associated Spring Raymond allows extension/retraction of the wheels; C: Three-dimensional printed ring (11.5 mm in diameter made of UV Curable Acrylic Plastic on a demo PillCam® SB2); D: Inserted into one end of a translucent tube and pulled through by a silk string.

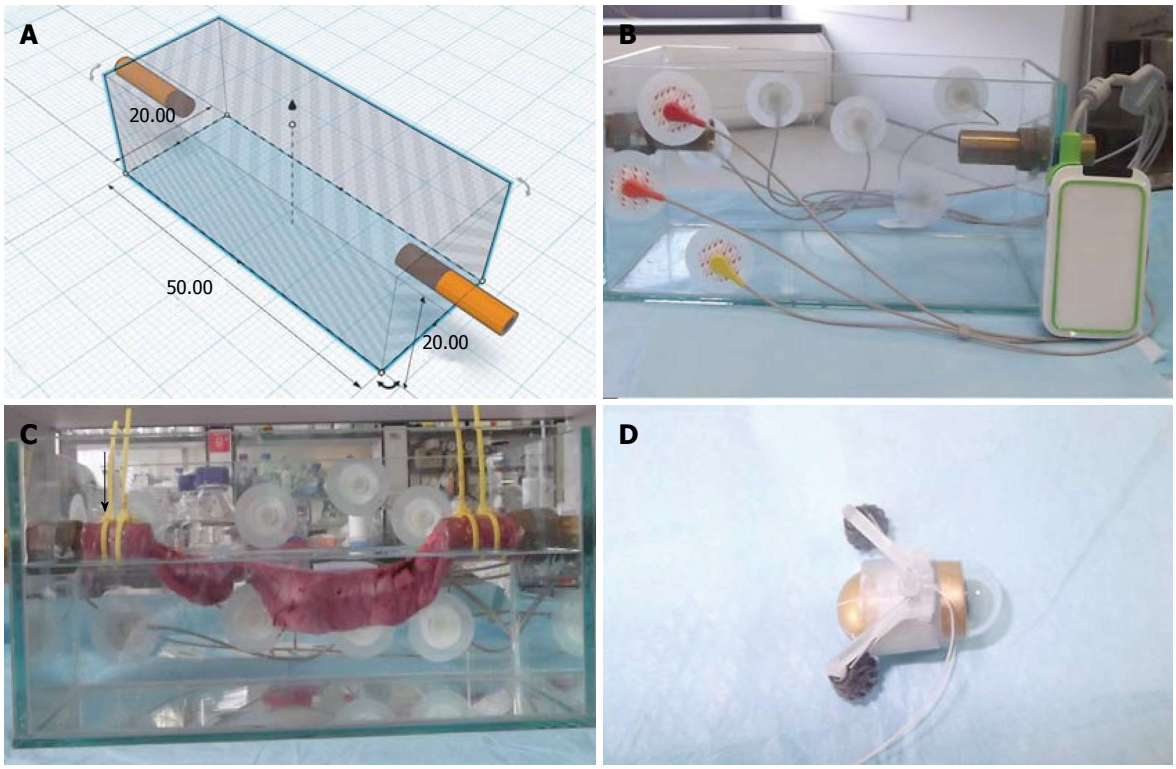

Figure 3 ***In vivo*** **experiment.** A and B: Glass tank (50 cm × 20 cm × 20 cm) with fix points for the intestine (metal tubes) and entry points for the capsule device, C: Standard simulated intestinal environment created by mounting 32 cm long, freshly harvested porcine small-intestine to both ends of a fluid-filled tank (arrow); D: Capsule device (assembled ring with wheels on spring on a MiroCam®) was inserted into the suspended bowel *via* one of the metal tubes.

systems (PillCam® SB2, Given® Imaging Ltd., Israel; MiroCam®, IntroMedic Ltd., South Korea) and the holes were used for the insertion of a 0.5 mm silk string.

For the *in vitro* experiment, a translucent polycarbonate tube (internal diameter 22 mm and 50 cm long; Figure 2D) was used. The tube characteristics mimic the diameter and slippery surface of an adult small-bowel. The assembled ring with wheels on spring was fitted on a demo PillCam® SB2 and was inserted into one end of the tube; thereafter, it was pulled through by the string (Figure 2D). While the capsule was pulled along the tube, the wheels were observed rotating constantly without any skidding effect. A video that demonstrates this experiment is available at: http://dl.dropbox.com/u/7591304/Capsule.mov.

For the *ex vivo* experiment; a glass tank (50 cm × 20 cm × 20 cm) with fix points for the intestine (metal tubes) and entry points for the assembled device was constructed (Figure 3A and B)[15]. A standard simulated intestinal environment (Figure 3C) was created by mounting a 32 cm long, freshly harvested porcine (Large White x Landrace 15-mo-old female sow) small-intestine to both ends of a fluid-filled tank[15]. The tank was filled with Normal Tyrode's Solution - Base1 (NTS-1) and Base2 (NTS-2), (Dr. Lohmann Diaclean GmbH, Germany), diluted with 9l of sterile water. Thereafter, the assembled ring with wheels on spring fitted on a MiroCam®) was inserted into the suspended bowel *via* one of the metal tubes (Figure 3D). Consistency of wheels' rotation was

validated by utilising an all-purpose endoscope, Findoo MircoCam (dnt® GmbH, Germany). The latter was introduced from the same end as the capsule and recorded the wheels movement while following the moving capsule. A video is available at: http://dl.dropboxusercontent.com/u/7591304/1035301R.AVI

In conclusion, *in vitro* and *ex vivo*, "proof of concept" experimentation based on a conceptual CE design (Figure 1A and B) - that at least in theory offers enhanced localisation capabilities - showed promising preliminary results. Further elaborate experiments (*i.e.*, at first stage, force measurements and construction of a functional prototype)[16] and *in vivo* experiments with this prototype are essential and currently under way.

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6

24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23243641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

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